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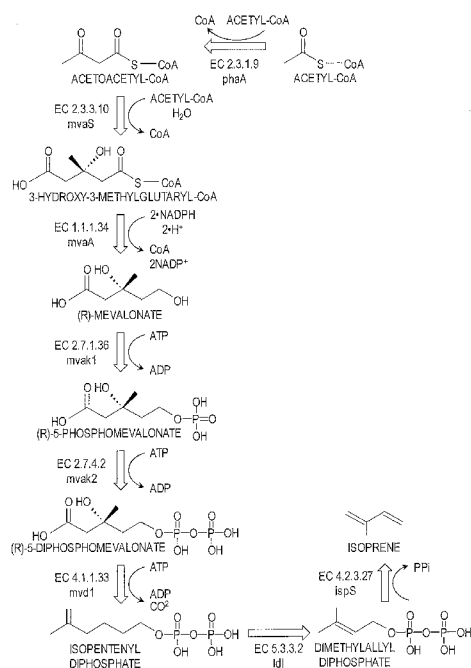


FIG. 3

(57) Abstract: The present invention relates to recombinant host cells polynucleotides and polypeptides, methods for their production, and methods for their use in production of hydrocarbons. Disclosed herein are methods, compositions and hosts for synthesizing hydrocarbons and derivatives thereof. In one non-limiting embodiment, the methods, compositions and hosts are used to synthesize hydrocarbons comprising one or more isoprene units as depicted in Formula I as well as salts or derivatives thereof. An aspect of the present invention relates to a genetically engineered host capable of producing hydrocarbons or derivatives thereof via a mevalonate (MVA) pathway.

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**Methods, Materials, Synthetic Hosts and Reagents for the
Biosynthesis of Hydrocarbons and Derivatives Thereof**

This patent application claims the benefit of priority
from U.S. Provisional Application Serial No. 62/527,595, filed
5 June 30, 2017, the teachings of which are herein incorporated
by reference in their entirety.

FIELD

The present invention relates to recombinant host cells
10 polynucleotides and polypeptides, methods for their
production, and methods for their use in production of
hydrocarbons.

BACKGROUND

15 Hydrocarbons are important monomers for the production of
specialty elastomers including motor mounts/fittings, surgical
gloves, rubber bands, golf balls and shoes. For example,
styrene-isoprene-styrene block copolymers form a key component
of hot-melt pressure-sensitive adhesive formulations and cis-
20 poly-isoprene is utilized in the manufacture of tires (Whited
et al. Industrial Biotechnology 2010 6(3):152-
163). Manufacturers of rubber goods depend on either imported
natural rubber from the Brazilian rubber tree or petroleum-
based synthetic rubber polymers (Whited et al. 2010, *supra*).

25 Given an over-reliance on petrochemical feedstocks,
biotechnology offers an alternative approach to the generation
of industrially relevant products, via biocatalysis.
Biotechnology offers more sustainable methods for producing
industrial intermediates, in particular isoprene and
30 isoprenoids.

Construction of recombinant microorganisms and methods of their use to produce hydrocarbons such as isoprene are known in the art. However, many of these methods and processes are unsatisfactory because they rely on agricultural commodities
5 such as sugar cane and corn which can be volatile in supply and cost from time to time. The use of sugar derived from agricultural waste, often called 'biomass' has been proposed.

There are known metabolic pathways leading to the synthesis of isoprene in eukaryotes such as *Populus alba* and
10 some prokaryotes such as *Bacillus subtilis* have been reported to emit isoprene (Whited et al. 2010, *supra*). Isoprene production in prokaryotes is however rare, and no prokaryotic isoprene synthase (hereafter ISPS) has been described to date.

Generally, two metabolic routes have been described
15 incorporating the molecule dimethylallyl-pyrophosphate, the precursor to isoprene. These are known as the mevalonate and the non-mevalonate pathways (Kuzuyama Biosci. Biotechnol. Biochem. 2002 66(8):1619-1627), both of which function in terpenoid synthesis *in vivo*. Both require the introduction of
20 a non-native ISPS in order to divert carbon to isoprene production.

The mevalonate pathway generally occurs in higher eukaryotes and Archaea and incorporates a decarboxylase enzyme, mevalonate diphosphate decarboxylase (hereafter MDD),
25 that introduces the first vinyl-group into the precursors leading to isoprene. The second vinyl-group is introduced by isoprene synthase in the final step in synthesizing isoprene. The non-mevalonate pathway or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway occurs in many bacteria
30 and dimethylallyl-PP is generated alongside isopentenyl-PP,

two molecules which are interconvertible via the action of isopentenyl pyrophosphate isomerase or isopentyl diphosphate isomerase (hereafter IDI).

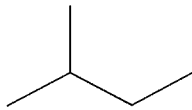
The mevalonate (MVA) dependent pathway can be split into
5 two units; the upper pathway for the generation of mevalonic acid from central metabolism and the lower pathway for the conversion of mevalonic acid to IPP and DMAPP. In the upper mevalonate pathway, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA by the action of acetoacetyl-CoA C-
10 acetyltransferase (AACT, EC 2.3.1.9). Acetoacetyl-CoA is then converted to HMG-CoA by HMG-CoA synthase (HMGS, EC 2.3.3.10) and HMG-CoA reductase (HMGR, EC 1.1.1.34) catalyses the reduction of HMG-CoA to mevalonate. Mevalonate then feeds into the lower mevalonate pathway where sequential
15 phosphorylation by mevalonate kinase (MVK, EC 2.7.1.36) and phosphomevalonate kinase (MPK, EC 2.7.4.2), followed by a decarboxylation reaction by mevalonate diphosphate decarboxylase (MDD, EC 4.1.1.33), converts mevalonate to IPP. IPP is then isomerised to DMAPP by isopentenyl diphosphate
20 isomerase (IDI, EC 5.3.3.2).

There is a need for genetically engineered hosts capable of stable hydrocarbon production.

SUMMARY

25 Disclosed herein are methods, compositions and hosts for synthesizing hydrocarbons and derivatives thereof.

In one nonlimiting embodiment, the methods, compositions and hosts are used to synthesize hydrocarbons comprising one or more isoprene units as depicted in Formula I

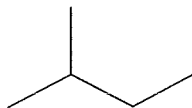


(I)

as well as salts or derivatives thereof.

An aspect of the present invention relates to a genetically engineered host capable of producing hydrocarbons
5 or derivatives thereof via a mevalonate (MVA) pathway.

In one nonlimiting embodiment the hydrocarbon produced from the genetically engineered host comprises one or more isoprene units as depicted in Formula I



(I)

10 or a salt or derivative thereof.

Recombinant hosts of the present invention comprise at least one genome-integrated synthetic operon encoding an enzyme of the MVA pathway.

In one nonlimiting embodiment, the genetically engineered
15 host comprises a genome-integrated synthetic operon encoding a plurality of enzymes of the MVA pathway.

In one nonlimiting embodiment, the genetically engineered host comprises a genome integrated synthetic operon encoding one or more enzymes of the upper MVA pathway.

20 In one nonlimiting embodiment, the genetically engineered host comprises a genome-integrated synthetic operon encoding the *Enterococcus faecalis* upper MVA pathway.

In one nonlimiting embodiment, the genetically engineered host comprises a genome integrated synthetic operon encoding
25 one or more enzymes of the lower MVA pathway.

In one nonlimiting embodiment, the genetically engineered host comprises a genome-integrated synthetic operon encoding *Streptococcus pneumoniae* lower MVA pathway.

In one nonlimiting embodiment, the genetically engineered
5 host comprises a genome-integrated synthetic operon encoding the *Enterococcus faecalis* upper MVA pathway and a genome-integrated synthetic operon encoding *Streptococcus pneumoniae* lower MVA pathway.

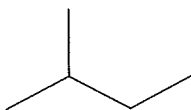
In one nonlimiting embodiment, the genetically engineered
10 host comprises a genome-integrated synthetic operon encoding mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD).

In any of these nonlimiting embodiments, the genetically engineered host may further comprise a genome-integrated
15 transcription unit coding for an isoprene synthase.

In any of these embodiments, the genetically engineered host may further comprise one or more plasmids encoding one or more enzymes of the upper and/or lower MVA pathways.

Another aspect of the present invention relates to a
20 method for producing genetically engineered stable strains of host cells which produce hydrocarbons.

In one nonlimiting embodiment the hydrocarbon produced from the genetically engineered host comprises one or more isoprene units as depicted in Formula I



25

(I)

or a salt or derivative thereof.

In one nonlimiting embodiment of this method, at least one synthetic operon encoding an enzyme of the MVA pathway is integrated into the host genome.

5 In one nonlimiting embodiment of this method, at least one synthetic operon encoding a plurality of enzymes of the MVA pathway is integrated into the host genome.

In one nonlimiting embodiment of this method, a synthetic operon encoding one or more enzymes of the upper MVA pathway is integrated into the host genome.

10 In one nonlimiting embodiment of this method, a synthetic operon encoding all or part of the *Enterococcus faecalis* upper MVA pathway is integrated into the host genome.

In one nonlimiting embodiment of this method, a synthetic operon encoding one or more enzymes of the lower MVA pathway
15 is integrated into the host genome.

In one nonlimiting embodiment of this method, a synthetic operon encoding all or part of the *Streptococcus pneumoniae* lower MVA pathway is integrated into the host genome.

In one nonlimiting embodiment, a synthetic operon
20 encoding all or part of the the *Enterococcus faecalis* upper MVA pathway and a synthetic operon encoding all or part of the *Streptococcus pneumoniae* lower MVA pathway are integrated into the host genome.

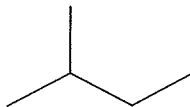
In one nonlimiting embodiment, a synthetic operon
25 encoding mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) is integrated into the host genome.

Any of these nonlimiting embodiments of this method may further comprise integrating a transcription unit coding for
30 an isoprene synthase into the host genome.

Further, in any of these embodiments, one or more plasmids encoding one or more enzymes of the upper and/or lower MVA pathways and/or an isoprene synthase may be incorporated into the host.

5 Another aspect of the present invention relates to methods for producing hydrocarbons in a genetically engineered host capable of stable hydrocarbon production.

In one nonlimiting embodiment the hydrocarbon produced from the genetically engineered host comprises one or more
10 isoprene units as depicted in Formula I



(I)

or a salt or derivative thereof.

Another aspect of the present invention relates to hydrocarbons such as bioderived hydrocarbons, produced in or
15 obtainable from a genetically engineered host capable of stable hydrocarbon production, such as a host as described herein.

Yet another aspect of the present invention relates to products such as bio-derived, bio-based, or fermentation-
20 derived products, produced from or obtainable from any of the hosts or methods described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention
25 pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other

references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are
5 illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and the
10 drawings, and from the claims. The word "comprising" in the claims may be replaced by "consisting essentially of" or with "consisting of," according to standard practice in patent law.

15 BRIEF DESCRIPTION OF THE FIGURES

FIGs. 1A through 1D are plasmid maps for integration vectors pTc(ColE1SacB)::*AphaB2C2*::P_{BAD}::*S. pneumoniae* L-MVA (FIG. 1A) and pTC(p15ASacB)::P_{CnBAD}::IspS401::TT::HMGR::AACT::P_{BAD} (FIG. 1B), *Salix* Sp. isoprene synthase expression plasmid
20 pISP407 (FIG. 1C) and DxpS/IspS expression plasmid pBBR1MCS3-pBAD-His-Bs_DXS_OPT-ISPS (FIG. 1D). Hatched arrows, open reading frames (ORFs); Black arrows, knockin vector homology arms; Cross-hatched arrows, araBAD promoter; IspS, isoprene synthase; TT, transcriptional terminator; Grey arrow, *E. coli*
25 origin of replication

FIGs. 2A and 2B shows *de novo* synthesis of isoprene and mevalonolactone in *C. necator* strains encoding genome-integrated gene copies of the *E. faecalis* upper MVA pathway and the *S. pneumoniae* lower MVA pathway. *C. necator* MVA
30 pathway strains and control strains were cultured at 30°C for

24 hours in 10 ml GC-MS vials containing 2 ml of defined media with different ammonium chloride concentrations. Isoprene was measured in the headspace and mevalonolactone was measured in the culture supernatant by GC-MS (n=3 except for bars with an asterix where n=2). Measurements were normalized to culture optical density (OD₆₀₀). Error bars represent Standard Deviation of the mean. ND, not done.

FIG. 3 is a schematic of the MVA pathway.

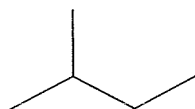
10 DETAILED DESCRIPTION

The present invention provides genetically engineered hosts capable of producing hydrocarbons and derivatives thereof via a mevalonate (MVA) pathway as well as methods for production of these hosts and methods for their use in production of hydrocarbons and derivatives thereof.

Accordingly, disclosed herein are recombinant cells comprising an engineered enzymatic pathway that catalyze the conversion of a gas to a hydrocarbon or derivative thereof, compositions and methods for their production, and methods for their use in production of hydrocarbons or derivatives thereof. The compositions and methods disclosed herein provide low cost processes for conversion of industrial gases to chemicals in a fermenter. In the methods of the present invention, recombinant cells are introduced into a fermenter, mixed with gas feedstocks which are enzymatically converted to a hydrocarbon by the recombinant cells, and the hydrocarbon is then separated from the off-gases from the fermenter.

By "recombinant cell" or "recombinant host" as used herein it is meant to encompass any genetically engineered cell or host as described herein and such terms as recombinant, engineered, and genetically engineered are used interchangeably
5 herein.

By "hydrocarbon" or hydrocarbons" as used herein, it is meant to encompass any organic compound comprised of carbons and hydrogens which can be enzymatically synthesized from a gas and is inclusive of saturated as well as unsaturated structures
10 with double or triple bonds formed between carbon atoms, ring structures, salts and derivatives thereof. In one nonlimiting embodiment, the hydrocarbon comprises one or more isoprene units as depicted in Formula I



(I)

15 or a salt or derivative thereof.

By the phrase "one or more isoprene units as depicted in Formula I" it is meant to encompass any saturated or unsaturated 5 carbon branched structure derived from an isoprenoid including, isoprene as well as isoprenoids,
20 terpenes and terpenoids as well as derivatives such as, but not limited to isoprenols, and salts thereof.

Nonlimiting examples of hydrocarbons comprising one or more isoprene units produced in accordance with the present invention include isoprene as well as any isoprenoid, terpene
25 or terpenoid derivative of 5, including C5, C10, C15, C20, C25, C30, C35, C40, C45, C50, etc. Nonlimiting examples include hemiterpene, monoterpene, diterpene, triterpene, tetraterpene, polyterpene, lycopene, abietadiene,

amorphadiene, carene, alpha-farnesene, beta-farnesene, farnesol, geraniol, geranylgeraniol, isoprene, linalool, limonene, myrcene, nerolidol, ocimene, patchoulol, beta-pinene, sabinene, gamma-terpinene, terpinolene and valencene,
5 as well as derivatives and salts thereof.

The mevalonate pathway for the conversion of acetyl-CoA to the isoprenoid precursors, IPP and DMAPP, is found in eukaryotes, archaea and some bacteria, but is absent from the facultative chemolithoautotrophic bacterium, *Cupriavidus*
10 *necator* (previously called *Hydrogenomonas eutrophus*, *Alcaligenes eutropha*, *Ralstonia eutropha*, and *Wautersia eutropha*). *Cupriavidus necator* is a Gram-negative, flagellated soil bacterium of the Betaproteobacteria class. This hydrogen-oxidizing bacterium is capable of growing at the
15 interface of anaerobic and aerobic environments and easily adapts between heterotrophic and autotrophic lifestyles. Sources of energy for the bacterium include both organic compounds and hydrogen. *C. necator* does not naturally contain genes for isoprene synthase (ISPS) and therefore does not
20 express this enzyme. Additional properties of *Cupriavidus necator* include microaerophilicity, copper resistance (Makar and Casida; 1987), bacterial predation (Byrd et al., 1985; Sillman & Casida, 1986; Zeph & Casida, 1986) and polyhydrobutyrate (PHB) synthesis. In addition, the cells have
25 been reported to be capable of both aerobic and nitrate dependent anaerobic growth.

In one nonlimiting embodiment, the host of the present invention is *Cupriavidus necator*, such as a genetically engineered strain of *Cupriavidus necator* capable of stable
30 hydrocarbon production. In one nonlimiting embodiment, the

present invention relates to *Cupriavidus necator* host capable of producing isoprenoids via a mevalonate (MVA) pathway, such as an isolated or substantially pure genetically engineered *Cupriavidus necator* host capable of producing isoprenoids via a mevalonate (MVA) pathway. A nonlimiting example of a *C. necator* host useful in the present invention is a *C. necator* of the H16 strain. In one nonlimiting embodiment, a *C. necator* host of the H16 strain with the *phaCAB* gene locus knocked out (Δ *phaCAB*) is used.

As used herein, a "substantially pure culture" of a recombinant host microorganism is a culture of that microorganism in which less than about 40% (i.e., less than about 35%; 30%; 25%; 20%; 15%; 10%; 5%; 2%; 1%; 0.5%; 0.25%; 0.1%; 0.01%; 0.001%; 0.0001%; or even less) of the total number of viable cells in the culture are viable cells other than the recombinant microorganism, e.g., bacterial, fungal (including yeast), mycoplasmal, or protozoan cells. The term "about" in this context means that the relevant percentage can be 15% of the specified percentage above or below the specified percentage. Thus, for example, about 20% can be 17% to 23%. Such a culture of recombinant microorganisms includes the cells and a growth, storage, or transport medium. Media can be liquid, semi-solid (e.g., gelatinous media), or frozen. The culture includes the cells growing in the liquid or in/on the semi-solid medium or being stored or transported in a storage or transport medium, including a frozen storage or transport medium. The cultures are in a culture vessel or storage vessel or substrate (e.g., a culture dish, flask, or tube or a storage vial or tube).

In another nonlimiting embodiment, the host of the present invention is a genetically engineered host having one or more of the above-mentioned properties of *Cupriavidus necator*.

5 In yet another nonlimiting embodiment, the host of the present invention is a genetically engineered host selected from non-pathogenic members of the genera *Ralstonia*, *Wausteria*, *Cupriavidus*, *Alcaligenes*, *Burkholderia* or *Pandoraea*.

10 The present invention provides methods and compositions for synthesizing hydrocarbons in these genetically engineered cells. In the methods and compositions of the present invention, a host of the invention, such as organisms such as *C. necator*, as well as non-pathogenic members of the genera
15 *Ralstonia*, *Wausteria*, *Alcaligenes*, *Burkholderia* and *Pandoraea*, and other organisms having one or more of the above-mentioned properties of *Cupriavidus necator* can be used to synthesize hydrocarbons via a mevalonate (MVA) dependent pathway.

Recombinant hosts of the present invention comprise at
20 least one genome-integrated synthetic operon encoding an enzyme of the MVA pathway. Nonlimiting examples of enzymes of this pathway include acetoacetyl-CoA C-acetyltransferase (AACT, EC 2.3.1.9) that catalyzes the chemical reaction
2 acetyl-CoA => CoA + acetoacetyl-CoA, HMG-CoA reductase (HMGR, EC 1.1.1.34) that catalyzes the reaction HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) => mevalonic acid, hydroxymethylglutaryl-CoA synthase (HMGS, EC 2.3.3.10) that catalyzes the reaction
25 acetoacetyl-CoA => HMG-CoA, mevalonate kinase (MVK, EC 2.7.1.36) that catalyzes the reaction mevalonic acid =>
30 mevalonate-5-phosphate, phosphomevalonate kinase (MPK, EC

2.7.4.2) that catalyzes the reaction mevalonate-5-phosphate => mevalonate-5-diphosphate, mevalonate diphosphate decarboxylase (MDD, EC 4.1.1.33) that catalyzes the reaction mevalonate-5-diphosphate => isopentyl-5-pyrophosphate, isopentenyl

5 diphosphate isomerase (IDI, EC 5.3.3.2) that catalyzes the reaction isopentyl-5-pyrophosphate => dimethylallyl pyrophosphate, and isoprene synthase (ISPS, EC 4.2.3.27) that catalyses the reaction dimethylallyl pyrophosphate => isoprene + diphosphate. The host may comprise any one or more of these
10 enzymes, such as at least two or at least three of these enzymes.

In one nonlimiting embodiment, the recombinant host comprises at least two genome-integrated synthetic operons encoding enzymes of the MVA pathway.

15 In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic operon encoding one or more enzymes of the upper MVA pathway into the host genome. In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic
20 operon encoding one or more enzymes of the *Enterococcus faecalis* upper MVA pathway into the host genome.

In one nonlimiting embodiment, the host genome comprises an operon encoding one or more enzymes of the *Enterococcus faecalis* upper MVA pathway. The operon may be a synthetic
25 operon. The operon may be exogenous to the host. One or more of the enzymes may be exogenous to the host. In some non-limiting embodiments, the operon encodes at least one AACT and/or at least one HMGR and/or at least one HMGS. In some non-limiting embodiments, the operon encodes at least one AACT
30 and at least one HMGR and at least one HMGS. In some non-

limiting embodiments, the operon includes sequences encoding AACT, HMGR and HMGS, arranged in that order.

Nonlimiting examples of enzymes of upper MVA pathway, genes of which can be integrated into the host genome, include
5 the polypeptides of AACT (e.g. SEQ ID NO:1 and SEQ ID NO:56), HMGR (e.g. SEQ ID NOs: 1, 58, 60, 62, 64 and 66) and HMGS (e.g. SEQ ID NOs:2, 68, 70, 72 and 74) as well as polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%
10 or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 1, 56, 58, 60, 62, 64, 66, 2, 68, 70, 72 or 74 or a functional fragment thereof. Nonlimiting examples of nucleic acid sequences encoding such enzymes which can be integrated into the host genome include the nucleic acid
15 sequences of AACT (e.g. SEQ ID NO:23 and SEQ ID NO:55), HMGR (e.g. SEQ ID NOs: 23, 57, 59, 61, 63 and 65) and HMGS (e.g. SEQ ID NO:24, 67, 69, 71 and 73) as well as nucleic acid sequences encoding polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%,
20 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NOs: 23, 55, 57, 59, 61, 63, 65, 24, 67, 69, 71 or 73 or a functional fragment thereof. The host may comprise any one or more of these enzymes, such as at least one AACT and/or at
25 least one HMGR and/or at least one HMGS. In some non-limiting embodiments, the host comprises at least one AACT and at least one HMGR and at least one HMGS. In one nonlimiting embodiment, the nucleic acid sequence is codon optimized for *C. necator* expression.

In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic operon encoding one or more enzymes of the lower MVA pathway into the host genome. In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic operon encoding one or more enzymes of the *Streptococcus pneumoniae* lower MVA pathway into the host genome.

In one nonlimiting embodiment, the host genome comprises an operon encoding one or more enzymes of the *Streptococcus pneumoniae* lower MVA pathway. The operon may be a synthetic operon. The operon may be exogenous to the host. One or more of the enzymes may be exogenous to the host. In some non-limiting embodiments, the operon encodes one or more MVK and/or one or more MPK and/or one or more MDD and/or one or more IDI. In some non-limiting embodiments, the operon encodes at least one MVK and at least one MPK and at least one MDD, or encodes at least one MVK and at least one MPK and at least one MDD and at least one IDI. In some non-limiting embodiments, the operon comprises sequences coding for MVK, MPK and MDD, arranged in that order.

Nonlimiting examples of enzymes of the lower MVA pathway, genes of which can be integrated into the host genome, include mevalonate kinase (MVK; e.g. SEQ ID NO:3), phosphomevalonate kinase (MPK; e.g. SEQ ID NO:4), mevalonate diphosphate decarboxylase (MDD; e.g. SEQ ID NO:5) and isopentenyl diphosphate isomerase (IDI; e.g. SEQ ID NO:6) as well as polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 3, 4, 5 or 6 or a functional

fragment thereof. Nonlimiting examples of nucleic acid sequences encoding such enzymes which can be integrated into the host genome include the nucleic acid sequences of MVK (e.g. SEQ ID NO:25), MPK (e.g. SEQ ID NO:26), MDD (e.g. SEQ ID NO:27) and IDI (e.g. SEQ ID NO:28) as well as nucleic acid sequences encoding polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NOs: 25, 26, 27 or 28 or a functional fragment thereof. The host may comprise any one or more of these enzymes, such as one or more MVK and/or one or more MPK and/or one or more MDD and/or one or more IDI. In some non-limiting embodiments, the host comprises at least one MVK and at least one MPK and at least one MDD, or encodes at least one MVK and at least one MPK and at least one MDD and at least one IDI.

In one nonlimiting embodiment, the nucleic acid sequence is codon optimized for *C. necator* expression.

In one nonlimiting embodiment, the genetically engineered strain comprises a genome-integrated synthetic operon encoding the one or more enzymes of the upper MVA pathway and a genome-integrated synthetic operon encoding one or more enzymes of the lower MVA pathway. In one nonlimiting embodiment, the genetically engineered strain comprises a genome-integrated synthetic operon encoding the one or more enzymes of the *Enterococcus faecalis* upper MVA pathway and a genome-integrated synthetic operon encoding one or more enzymes of the *Streptococcus pneumoniae* lower MVA pathway.

In one non-limiting embodiment, the host comprises any one or more enzymes of the upper MVA pathway as described

above and any one or more enzymes of the lower MVA pathway as described above. In some non-limiting embodiments, these enzymes of the upper and/or lower MVA pathway are integrated into the genome of the host.

5 In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic operon encoding mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) into the host genome. Nonlimiting examples of mevalonate kinase (MK),
10 mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) enzymes, genes of which can be integrated into the host genome, include the polypeptides of SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 as well as polypeptides with similar enzymatic activities exhibiting at
15 least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 or a functional fragment thereof. Nonlimiting examples of nucleic acid sequences encoding such
20 enzymes which can be integrated into the host genome include the nucleic acid sequences of SEQ ID NO:s 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44 as well as nucleic acid sequences encoding polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%,
25 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NOs: 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44 or a functional fragment thereof.

In one nonlimiting embodiment, the nucleic acid sequence
30 is codon optimized for *C. necator* expression.

In one nonlimiting embodiment, the genetically engineered strain is produced by integration of a synthetic operon comprising SEQ ID NO: 48, 49 or 50.

In some nonlimiting embodiments, the recombinant host further comprises at least one additional plasmid encoding an enzyme of the MVA pathway.

In some nonlimiting embodiments, the recombinant host further comprises a plurality of plasmids expressing enzymes of the lower and/or upper MVA pathway.

10 In some nonlimiting embodiments, the genetically engineered strain may further comprise a sequence coding for an isoprene synthase enzyme, such as a genome-integrated transcription unit coding for an isoprene synthase enzyme. Nonlimiting examples of isoprene synthase enzymes include the
15 polypeptide sequence of *Populus alba* isoprene synthase (IspS), EC 4.2.3.27 (accession number Q50L36; SEQ ID NO:7), and the polypeptide sequence of *Salix* sp. DG-2011 isoprene synthase (IspS), EC 4.2.3.27 (accession number AEK70970; SEQ ID NO:8) as well as polypeptides with similar enzymatic activities
20 exhibiting at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 7 or 8 or a functional fragment thereof. Nonlimiting examples of nucleic acid sequences encoding such enzymes which can be integrated into
25 the host genome include the nucleic acid sequences of *Populus alba* isoprene synthase (SEQ ID NO:29) and *Salix* sp. Isoprene synthase (SEQ ID NO:30) as well as nucleic acid sequences encoding polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%,
30 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the

nucleic acid sequence set forth in SEQ ID NOs: 29 or 30 or a functional fragment thereof.

In one nonlimiting embodiment, the nucleic acid sequence is codon optimized for *C. necator* expression.

5 Integration of the transcription unit coding for an isoprene synthase enzyme provides a convenient readout for DMAPP production in the host.

The percent identity (homology) between two amino acid sequences as disclosed herein can be determined as follows.

10 First, the amino acid sequences are aligned using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLAST containing BLASTP version 2.0.14. This stand-alone version of BLAST can be obtained from the U.S. government's National Center for Biotechnology Information web site (www

15 with the extension ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two amino acid sequences using the BLASTP algorithm. To compare two amino acid sequences, the options of Bl2seq are set as

20 follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options

25 are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq-i c:\seq1.txt-j c:\seq2.txt-p blastp-o c:\output.txt. If the two compared sequences share homology (identity), then the

30 designated output file will present those regions of homology

as aligned sequences. If the two compared sequences do not share homology (identity), then the designated output file will not present aligned sequences. Similar procedures can be following for nucleic acid sequences except that blastn is
5 used.

Once aligned, the number of matches is determined by counting the number of positions where an identical amino acid residue is presented in both sequences. The percent identity (homology) is determined by dividing the number of matches by
10 the length of the full-length polypeptide amino acid sequence followed by multiplying the resulting value by 100. It is noted that the percent identity (homology) value is rounded to the nearest tenth. For example, 90.11, 90.12, 90.13, and 90.14 is rounded down to 90.1, while 90.15, 90.16, 90.17, 90.18, and
15 90.19 is rounded up to 90.2. It also is noted that the length value will always be an integer.

It will be appreciated that a number of nucleic acids can encode a polypeptide having a particular amino acid sequence. The degeneracy of the genetic code is well known to the art;
20 i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. For example, codons in the coding sequence for a given enzyme can be modified such that optimal expression in a particular species (e.g., bacteria or fungus) is obtained, using
25 appropriate codon bias tables for that species.

Functional fragments of any of the polypeptides or nucleic acid sequences described herein can also be used in the methods of the document. The term "functional fragment" as used herein refers to a peptide fragment of a polypeptide or a
30 nucleic acid sequence fragment encoding a peptide fragment of

a polypeptide that has at least 25% (e.g., at least: 30%; 40%; 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 98%; 99%; 100%; or even greater than 100%) of the activity of the corresponding mature, full-length, polypeptide. The functional fragment can
5 generally, but not always, be comprised of a continuous region of the polypeptide, wherein the region has functional activity.

Methods of the present invention can be performed in a host as described herein, such as a recombinant *Cupriavidus*
10 *necator* host, as well as non-pathogenic members of the genera *Ralstonia*, *Wausteria*, *Alcaligenes*, *Burkholderia* and *Pandoraea*, and other organisms having one or more of the above-mentioned properties of *Cupriavidus necator*.

Recombinant hosts can naturally express none or some
15 (e.g., one or more, two or more) of the enzymes of the pathways described herein. Endogenous genes of the recombinant hosts also can be disrupted to prevent the formation of undesirable metabolites or prevent the loss of intermediates in the pathway through other enzymes acting on such
20 intermediates. Recombinant hosts can be referred to as recombinant host cells, engineered cells, genetically engineered cells, genetically engineered hosts or engineered hosts. Thus, as described herein, recombinant hosts can include exogenous nucleic acids encoding one or more of
25 enzymes of the MVA pathway, as described herein. The recombinant host of the invention may be any genetically engineered cell or host as described herein.

The term "exogenous" as used herein with reference to a nucleic acid (or a protein) and a host refers to a nucleic
30 acid that does not occur in (and cannot be obtained from) a

cell of that particular type as it is found in nature or a protein encoded by such a nucleic acid. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a host once in the host. It is important to note that non-naturally-occurring nucleic acids can contain nucleic acid subsequences or fragments of nucleic acid sequences that are found in nature provided the nucleic acid as a whole does not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a host cell once introduced into the host, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid. A nucleic acid that is naturally-occurring can be exogenous to a particular host microorganism. For example, an entire chromosome isolated from a cell of yeast x is an exogenous nucleic acid with respect to a cell of yeast y once that chromosome is introduced into a cell of yeast y.

In contrast, the term "endogenous" as used herein with reference to a nucleic acid (e.g., a gene) (or a protein) and a host refers to a nucleic acid (or protein) that does occur in (and can be obtained from) that particular host as it is found in nature. Moreover, a cell "endogenously expressing" a nucleic acid (or protein) expresses that nucleic acid (or protein) as does a host of the same particular type as it is found in nature. Moreover, a host "endogenously producing" or that "endogenously produces" a nucleic acid, protein, or other compound produces that nucleic acid, protein, or compound as does a host of the same particular type as it is found in nature.

The utility of the MVA pathway for stable hydrocarbon production in *Cupriavidus necator* was evaluated in a genetically engineered strain containing two genome-integrated synthetic operons encoding the *Enterococcus faecalis* upper MVA pathway and *Streptococcus pneumoniae* lower MVA pathway, with a third genome-integrated transcription unit coding for the *Populus alba* isoprene synthase (IspS, EC 4.2.3.27) to provide a convenient readout for DMAPP production.

The *E. faecalis* upper MVA pathway and the *S. pneumoniae* lower MVA pathway were integrated into the *C. necator* H16 genome by homologous recombination as two arabinose-inducible operons, together with a third synthetic gene encoding *P. alba* isoprene synthase to provide a readout for DMAPP production. The resulting strain, BDISC_0921, was transformed with plasmid pISP407 expressing a second isoprene synthase gene from *Salix* Sp., to ensure there was sufficient *in vivo* isoprene activity to draw carbon flux through the MVA pathway. BDISC_0921 and its plasmid-containing derivative, BDISC_0990, were tested in an

isoprene/mevalonolactone bioassay against a *C. necator* strain with enhanced DMAPP production through the overexpression of the non-mevalonate pathway enzyme, DxpS, and a control strain, BDISC_0913, containing only the upper MVA pathway with the *P. alba* IspS.

To create a utilizable pool of acetyl-CoA for the mevalonate pathway, the isoprene and mevalonolactone bioassays were performed under nutrient-limitation conditions to induce the *C. necator* stringent response (Brigham et al. 2012).

Following induction of the MVA/MEP pathway enzymes with arabinose, the strains were cultured for 24 hours in a titration series of defined media containing progressively lower concentrations of the main nitrogen source, ammonium chloride (See FIG. 2A). Of the strains tested, the two MVA pathway strains, BDISC_0921 and BDISC_0990, produced the highest isoprene titers of 0.63 and 1.76 mg/L/OD₆₀₀, respectively, in media with the lowest ammonium chloride concentration of 0.2 g/L (see FIG. 2A). By contrast, isoprene production from the MEP pathway strain, BDISC_0664, was significantly lower, ranging from 0.05-0.23 mg/L/OD₆₀₀ (see FIG. 2A). Mevalonolactone was only detectable above 1 mg/L in BDISC_0913, containing a genome-integrated copy of the upper MVA pathway in the absence of the mevalonate-consuming lower MVA pathway (see FIG. 2B). As with isoprene production in the full MVA pathway strains, BDISC_0913 produced the highest mevalonolactone titre, of 4.5 mg/L/OD₆₀₀, with 0.2 g/L ammonium chloride. Both the isoprene and mevalonolactone assay results are consistent with higher carbon flux going through the MVA pathway under nitrogen-limitation conditions used to induce the *C. necator* stringent response. Taken together these

results confirm that the genome-integrated gene copies of the *E. faecalis* upper MVA pathway and the *S. pneumoniae* lower MVA pathway are functional in *C. necator* and are able to support the detectable bioconversion of acetyl-CoA from central metabolism to isoprene.

Further, several 1-MVA variants were identified as being particularly effective in producing hydrocarbons as well. Nucleic acid sequences used in identifying these variants include SEQ ID NO: 45, 46 and 47. The identified 1-MVA variants comprise the following combination of genes.

	MK	MPK	MDD
1-MVA-9	<i>M. mazei</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
1-MVA-11	<i>S. pneumoniae</i>	<i>S. pneumoniae</i>	<i>S. pneumoniae</i>
1-MVA-12	<i>S. pneumoniae</i>	<i>Lactococcus lactis</i>	<i>S. pneumoniae</i>

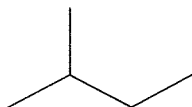
The full sequences of the operons for 1-MVA-9, 1-MVA-11 and 1-MVA-12 are depicted in SEQ ID NO:s 48, 49 and 50, respectively.

Thus, the present invention also provides methods for hydrocarbon production.

In a non-limiting embodiment, a method of hydrocarbon production as described herein is carried out using any host of the invention.

In a non-limiting embodiment is provided the use of a host of the invention, such as a recombinant or genetically engineered host as described herein, for the production of a hydrocarbon. The use may involve any method of hydrocarbon production as described herein.

In one nonlimiting embodiment the hydrocarbon produced from the genetically engineered host comprises one or more isoprene units as depicted in Formula I



(I)

5 or a salt or derivative thereof.

Methods of the present invention can be performed in a recombinant *Cupriavidus necator* host, as well as non-pathogenic members of the genera *Ralstonia*, *Wauteria*, *Alcaligenes*, *Burkholderia* and *Pandoraea*, and other organisms
10 having one or more of the above-mentioned properties of *Cupriavidus necator*.

In one nonlimiting embodiment, the method comprises enzymatically converting mevalonate to isopentenylpyrophosphate in a genetically engineered host as described
15 herein.

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host comprising at least one genome-integrated synthetic operon encoding an enzyme of the MVA pathway. Nonlimiting examples
20 of enzymes of this pathway include acetoacetyl-CoA C-acetyltransferase (AACT), HMG-CoA reductase (HMGR), hydroxymethylglutaryl-CoA synthase (HMGS), mevalonate kinase (MVK), phosphomevalonate kinase (MPK), mevalonate diphosphate decarboxylase (MDD), isopentenyl diphosphate isomerase (IDI) and
25 isoprene synthase (ISPS).

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host

comprising at least two genome-integrated synthetic operons encoding enzymes of the MVA pathway.

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host

5 comprising a synthetic operon encoding one or more enzymes of the upper MVA pathway into the host genome. In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host comprising a synthetic operon encoding one or more enzymes of the *E. faecalis*, *C.*

10 *necator*, *S. cerevisiae*, *L. monocytogenes*, *S. pneumonia*, *L. lactis* or *S. aureus* upper MVA pathway into the host genome.

In this embodiment, any of the nucleic acid sequences encoding a polypeptide having AACT (e.g. SEQ ID NO:1 and SEQ ID NO:56), HMGR (e.g. SEQ ID NOS: 1, 58, 60, 62, 64 and 66) and HMGS

15 (e.g. SEQ ID NOS:2, 68, 70, 72 and 74) as well as polypeptides with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOS: 1, 56, 58, 60, 62, 64, 66, 2, 68, 70, 72 or 74

20 or a functional fragment thereof. as described *supra* can be used. Nonlimiting examples of nucleic acid sequences encoding such enzymes which can be integrated into the host genome

include the nucleic acid sequences of AACT (e.g. SEQ ID NO:23 and SEQ ID NO:55), HMGR (e.g. SEQ ID NOS: 23, 57, 59, 61, 63

25 and 65) and HMGS (e.g. SEQ ID NO:24, 67, 69, 71 and 73) as well as nucleic acid sequences encoding polypeptides with

similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in

30

SEQ ID NOs: 23, 55, 57, 59, 61, 63, 65, 24, 67, 69, 71 or 73
or a functional fragment thereof.

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host
5 comprising a synthetic operon encoding one or more enzymes of the lower MVA pathway into the host genome. In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered host comprising a synthetic operon encoding one or more enzymes of the *Streptococcus*
10 *pneumoniae* lower MVA pathway into the host genome. In this embodiment, any of the nucleic acid sequences encoding a polypeptide having mevalonate kinase (MVK; e.g. SEQ ID NO:3), phosphomevalonate kinase (MPK; e.g. SEQ ID NO:4), mevalonate diphosphate decarboxylase (MDD; e.g. SEQ ID NO:5) or
15 isopentenyl diphosphate isomerase (IDI; e.g. SEQ ID NO:6) activity or a polypeptide with similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 3, 4, 5 or 6 or a
20 functional fragment thereof as described *supra* can be used.

In another nonlimiting embodiment, the method is performed using a genetically engineered host comprising one or more exogenous nucleic acid sequences encoding one or more enzymes of the upper MVA pathway and one or more exogenous
25 nucleic acid sequences encoding one or more enzymes of the lower MVA pathway. In one nonlimiting embodiment, the method is performed using a genetically engineered host comprising one or more exogenous nucleic acid sequences encoding one or more enzymes of the *Enterococcus faecalis* upper MVA pathway as
30 described *supra* and one or more exogenous nucleic acid

sequences encoding one or more enzymes of the *Streptococcus pneumoniae* lower MVA pathway as described supra.

In another nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered strain produced by integration of a synthetic operon encoding mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) into the host genome. In this embodiment, any of the nucleic acid sequences encoding a mevalonate kinase (MK), mevalonate phosphokinase (MPK), and/or mevalonate decarboxylase (MDD) enzyme including the polypeptides of SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 or polypeptides exhibiting similar enzymatic activities exhibiting at least 70%, 75%, 80, 85, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 or a functional fragment thereof as described supra can be used.

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered strain comprising a nucleic acid sequence codon optimized for *C. necator* expression.

In one nonlimiting embodiment, the hydrocarbon production method is performed using a genetically engineered strain comprising a synthetic operon comprising SEQ ID NO: 48, 49 or 50.

In some nonlimiting embodiments, the hydrocarbon production method is performed using a genetically engineered strain further comprising at least one additional plasmid encoding an enzyme of the MVA pathway.

In some nonlimiting embodiments, the hydrocarbon production method is performed using a genetically engineered strain further comprising a plurality of plasmids expressing enzymes of the lower and/or upper MVA pathway.

5 In some nonlimiting embodiments, the genetically engineered strain used in the hydrocarbon production methods may further comprise a genome-integrated transcription unit coding for an isoprene synthase enzyme as described supra.

10 In some non-limiting embodiments, the method comprises contacting a host of the invention with a suitable substrate under conditions such that the host is capable of producing a hydrocarbon from the substrate via a MVA pathway. Such a method may comprise culturing a host of the invention in the presence of a suitable substrate. In some non-limiting
15 embodiments, the substrate is acetyl-CoA, or a substrate that can be converted to form acetyl-CoA by the host.

In any the methods described herein, a fermentation strategy can be used that entails anaerobic, micro-aerobic or aerobic cultivation. A fermentation strategy can entail
20 nutrient limitation such as nitrogen, phosphate or oxygen limitation. A cell retention strategy using a ceramic hollow fiber membrane can be employed to achieve and maintain a high cell density during fermentation. The substrate, or the principal carbon source fed to the fermentation, can be or can
25 derive from a biological or non-biological feedstock. The biological feedstock can be, or can derive from, monosaccharides, disaccharides, lignocellulose, hemicellulose, cellulose, lignin, levulinic acid and formic acid, triglycerides, glycerol, fatty acids, agricultural waste,
30 condensed distillers' solubles or municipal waste. The non-

biological feedstock can be, or can derive from, natural gas, syngas, CO₂/H₂, methanol, ethanol, non-volatile residue (NVR) a caustic wash waste stream from cyclohexane oxidation processes or waste stream from a chemical industry such as, but not
5 limited to a carbon black industry or a hydrogen-refining industry, or petrochemical industry.

In one nonlimiting embodiment, at least one of the enzymatic conversions of the hydrocarbon production method comprises gas fermentation within the recombinant *Cupriavidus*
10 *necator* host, or a non-pathogenic member of the genera *Ralstonia*, *Wausteria*, *Alcaligenes*, *Burkholderia* and *Pandoraea*, and other recombinant organism having one or more of the above-mentioned properties of *Cupriavidus necator*. In this embodiment, the gas fermentation may comprise at least one of
15 natural gas, syngas, CO₂/H₂, methanol, ethanol, non-volatile residue, caustic wash from cyclohexane oxidation processes, or waste stream from a chemical industry such as, but not limited to a carbon black industry or a hydrogen-refining industry, or petrochemical industry. In one nonlimiting embodiment, the
20 gas fermentation comprises CO₂/H₂.

In some non-limiting embodiments the substrate is a gas. In some non-limiting embodiments, the method comprises contacting the host with a gas. In some of these embodiments, the gas comprises at least one of natural gas, syngas, CO₂/H₂,
25 methanol, ethanol, non-volatile residue, caustic wash from cyclohexane oxidation processes, or waste stream from a chemical industry such as, but not limited to a carbon black industry or a hydrogen-refining industry, or petrochemical industry. In one non-limiting embodiment, the gas comprises
30 CO₂/H₂.

The methods of the present invention may further comprise recovering produced hydrocarbons from the recombinant host. In these embodiments, the hydrocarbons may comprise one or more isoprene units, as described herein.

5 In some non-limiting embodiments, the hydrocarbon produced by a method of the invention is a gas. In some non-limiting embodiments the method further comprises recovering produced gaseous hydrocarbon from the host.

 Once produced, any method can be used to isolate
10 hydrocarbons. For example, hydrocarbons can be recovered from the fermenter off-gas stream as a volatile product as the boiling point of isoprene is 34.1°C. At a typical fermentation temperature of approximately 30°C., hydrocarbons have a high vapor pressure and can be stripped by the gas flow rate
15 through the broth for recovery from the off-gas. Hydrocarbons can be selectively adsorbed onto, for example, an adsorbent and separated from the other off-gas components. Membrane separation technology may also be employed to separate hydrocarbons from the other off-gas compounds. Hydrocarbons
20 may be desorbed from the adsorbent using, for example, nitrogen and condensed at low temperature and high pressure.

 Because of the gaseous nature of isoprene, in embodiments of the present invention wherein the hydrocarbon produced is isoprene, an advantage is easy separation of the product.

25 Also provided by the present invention are hydrocarbons bioderived from, produced by, or obtainable from, a recombinant host according to any of methods described herein. In one nonlimiting embodiment, the hydrocarbon has carbon isotope ratio that reflects an atmospheric carbon dioxide
30 uptake source. Examples of such ratios include, but are not

limited to, carbon-12, carbon-13, and carbon-14 isotopes.

In addition, the present invention provides a product such as a bio-derived, bio-based, or fermentation-derived product produced using the methods and/or compositions
5 disclosed herein. Examples of such products include, but are not limited to, compositions comprising at least one bio-derived, bio-based, or fermentation-derived compound or any combination thereof, as well as polymers, rubbers such as cis-polyisoprene rubber, trans-polyisoprene rubber, or liquid
10 polyisoprene rubber, molded substances, formulations and semi-solid or non-solid streams comprising one or more of the bio-derived, bio-based, or fermentation-derived compounds or compositions, combinations or products thereof.

In addition, the present invention provides methods of
15 producing such a product. In some non-limiting embodiments, the method comprises producing a hydrocarbon by a method of the invention and converting the hydrocarbon to said product. In one non-limiting embodiment, a method of producing a polymer comprises the steps of producing a hydrocarbon by a
20 method as described herein and forming a polymer from said hydrocarbon.

Although specific advantages have been enumerated above, various embodiments may include some, none, or all of the enumerated advantages. Further, other technical advantages
25 may become readily apparent to one of ordinary skill in the art after review of the figures and description herein. It should be understood at the outset that, although exemplary embodiments are illustrated in the figures and described herein, the principles of the present disclosure may be
30 implemented using any number of techniques, whether currently

known or not. The present disclosure should in no way be limited to the exemplary implementations and techniques illustrated in the drawings and described herein.

Unless otherwise specifically noted, articles depicted in the drawings are not necessarily drawn to scale.

Modifications, additions, or omissions may be made to the systems, apparatuses, and methods described herein without departing from the scope of the disclosure. For example, the components of the systems and apparatuses may be integrated or separated. Moreover, the operations of the systems and apparatuses disclosed herein may be performed by more, fewer, or other components and the methods described may include more, fewer, or other steps. Additionally, steps may be performed in any suitable order. As used in this document, "each" refers to each member of a set or each member of a subset of a set.

To aid the Patent Office and any readers of any patent issued on this application in interpreting the claims appended hereto, applicants wish to note that they do not intend any of the appended claims or claim elements to invoke 35 U.S.C. 112(f) unless the words "means for" or "step for" are explicitly used in the particular claim.

The following section provides further illustration of the methods and compositions of the present invention. These working examples are illustrative only and are not intended to limit the scope of the invention in any way.

Examples

Example 1: Gene Selection

The MVA pathway was assembled in *C. necator* from genes encoding the enzymes of the *E. faecalis* upper MVA pathway (AACT and HMGR) and *S. pneumoniae* lower MVA pathway (MVK, MPK, MDD and IDI). The performance of the *E. faecalis/S. pneumoniae* mevalonate pathway was monitored in live *C. necator* cells by converting DMAPP to isoprene with truncated versions of the *Populus alba* and *Salix* sp. DG-2011 isoprene synthase (IspS, EC 4.2.3.27), each containing a deletion of residues 1 to 36 encoding plastidic targeting sequences. Synthetic genes encoding these enzymes were codon optimized for expression in *C. necator* (see polypeptide and nucleotide sequences in Appendices 1 and 2).

Example 2: Construction of the *C. necator* genome-integrated MVA pathway strain

Two knockin vectors were assembled for the targeted integration of the *E. faecalis* upper MVA pathway, the *S. pneumoniae* lower MVA pathway and *P. alba* isoprene synthase into the *C. necator* genome. Plasmid pTc(ColE1SacB):: Δ *phaB2C2*::P_{BAD}::*S. pneumoniae* L-MVA (FIG. 1A) consisted of a synthetic operon encoding the *S. pneumoniae* lower MVA pathway (MVK, MPK, MDD) under the control of the araBAD promoter, flanked by 1 kb homology arms to the surrounding genes of *phaB2C2* locus. When introduced into *C. necator* H16 by conjugation, the knockin vector was designed to integrate the lower MVA pathway at the *phaB2C2* locus of chromosome 1 by homologous recombination and delete the *phaB2C2* operon. The second knockin vector,

pTC(p15ASacB):: Δ phaCAB::P_{CnBAD}::IspS401::TT::HMGR::AACT::P_{BAD}
 (FIG. 1B), consisted of separate transcription units coding
 for the *E. faecalis* upper MVA pathway and *P. alba* isoprene
 synthase under the control two facing P_{BAD} promoters, flanked
 5 by 1.5kb homology arms to the *phaC1AB1* locus. The *P. alba*
 IspS/*E. faecalis* upper MVA pathway double promoter cassette
 was designed to integrate at the *phaC1AB1* locus on chromosome
 1 and delete the *phaC1AB1* operon encoding essential enzymes of
 the competing polyhydroxyalkanoate (PHA) pathway.

10 The knockin vectors were introduced into *C. necator* H16
 by conjugation, essentially as described by Slater *et al.*,
 1998. *C. necator* exconjugants derived from single cross-over
 events were selected on defined medium (1.15 g/L KH₂PO₄; 1.15
 g/L Na₂HPO₄; 1 g/L NH₄Cl; 0.5 g/L MgSO₄·7H₂O; 0.062 g/L
 15 CaCl₂·2H₂O; 2 g/L fructose; 15 mg/L FeSO₄·7H₂O; 2.4 mg/L
 MnSO₄·H₂O; 2.4 mg/L ZnSO₄·7H₂O; 0.48 mg/L CuSO₄·5H₂O)
 supplemented with 20µg/ml tetracycline. Marker recovery was
 then performed by sacB counter-selection on LB agar plates
 supplemented with 10% w/v sucrose and 2% w/v fructose. For the
 20 construction of the genome-integrated MVA pathway strain, the
S. pneumoniae lower MVA pathway was first integrated into wild
 type *C. necator* H16. Following marker recovery, sucrose-
 resistant colonies were genotyped by colony PCR with primers
 BDIPRIM 4874 and 4875 (Table 1), to confirm the correct
 25 insertion of the P_{BAD}::L-MVA pathway operon at the *phaB2C2*
 locus. A PCR-positive clone containing the Δ phaC1AB1::P_{BAD}::L-
 MVA pathway insertion was then mated with an *E. coli* S17-1
 donor strain containing plasmid
 pTC(p15ASacB):: Δ phaCAB::P_{CnBAD}::IspS401::TT:: HMGR::AACT::P_{BAD}.
 30 Following a second round of sacB counter-selection, sucrose-

resistant colonies displaying a PHA-deficient visual phenotype were genotyped by colony PCR with primers BDIPRIM 2496 and 2543 (Table 1), to confirm the correct insertion of the IspS/U-MVA pathway cassette at the *phaC1AB1* locus. The resulting genome-integrated MVA pathway strain, BDISC_0921, was evaluated against two control strains, BDISC_0913 containing just the *E. faecalis* upper MVA pathway and IspS cassette integrated at the *phaC1AB1* locus and a plasmid-based strain, BDISC_0664 (*C. necator* H16 Δ *phaC1AB1*::pBBR1MCS3-pBAD-His-Bs_DXS_OPT-ISPS, FIG. 1D) expressing *P. alba* IspS and *Bacillus subtilis* MEP pathway enzyme 1-deoxy-D-xylulose-5-phosphate synthase, DxpS (EC 2.2.1.7), from an arabinose-inducible promoter. To increase isoprene titers from the genome-integrated MVA pathway, plasmid pISP407 (FIG. 1C), expressing a second arabinose-inducible isoprene synthase (*Salix* Sp. DG-2011 IspS), was introduced into BDISC_0921 by electroporation, creating *C. necator* strain BDISC_0990 (BDISC_0921::pISP407).

Example 3: Primers

Table 1 provides the amplification primers used for genotyping *C. necator* H16 Δ *phaB2C2* and Δ *phaC1AB1* gene knockin strains.

TABLE 1:

Primer No	Description		Primer Sequence
BDIPRIM4874	<i>C. necator</i> H16 Δ <i>phaB2C2</i>	F	GAGAGCCGGCTGACATAGAC (SEQ ID NO:51)
BDIPRIM4875	Knockout/Knockin genotyping primers	R	CGGCGACCTATGAGATCACT (SEQ ID NO:52)

BDIPRIM2496	<i>C. necator</i> H16 <i>ΔphaC1AB1</i>	F	AGAAGGCTGGGACGAAGTCT (SEQ ID NO:53)
BDIPRIM2543	Knockout/Knockin genotyping primers	R	CAAATTTCCGACCGCTGGTATTC (SEQ ID NO:54)

Example 4: De novo isoprene and mevalonate production from the *C. necator* genome-integrated MVA pathway strain

The genome-integrated MVA pathway strains, BDISC_0921 and
5 BDISC_0990 (BDISC_0921::pISP407), were evaluated for *de novo*
isoprene and mevalonolactone synthesis against control strains
BDISC_0913 and BDISC_0664 in bioassays based on gas
chromatography-mass spectrometry (GC-MS). To maximize the
carbon flux into the MVA pathway, the isoprene and
10 mevalonolactone bioassays were performed in defined media
under nitrogen limitation conditions similar to those used for
PHA production (Ishizaki, 2001). The optimal carbon to
nitrogen ratio for isoprene/mevalonolactone production was
tested by performing the bioassays in defined media with
15 different ammonium chloride concentrations, ranging from 0.2
to 1.0 g/L.

Seeding cultures were prepared for each strain by
inoculating a single colony into 20 ml of 27.5 g/L Tryptone
Soya broth without Dextrose (TSB-D media, Sigma Aldrich
20 catalogue number T3938-500G) containing the appropriate
antibiotic when necessary. The seeding cultures were incubated
at 30°C, 230rpm for 48 hours, then diluted by 1 in 50 into
fresh TSB-D media (10 ml) in 50 ml sterile Falcon tubes and
incubated for approximately 6 hours at 30°C, 230rpm. MVA
25 pathway and isoprene synthase expression was induced by adding
arabinose to a final concentration of 1 % w/v and the cultures
were incubated for a further 16 hours (overnight) at 30°C,

230rpm. The cultures were pelleted by centrifugation at 6000 g for 20 minutes and wet cell weight was measured for each cell pellet. The density of each culture was normalized to 0.1 g WCW/ml by re-suspending the *C. necator* cells with the appropriate volume of defined media (1.15 g/L KH_2PO_4 ; 1.15 g/L Na_2HPO_4 ; 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.062 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 2 g/L fructose; 15 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 2.4 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 2.4 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.48 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) containing each of the NH_4Cl concentrations tested (0.2, 0.4, 0.6, 0.8, 1 g/L). For each experimental variable (strain and NH_4Cl concentration), separate isoprene and mevalolactone bioassays were set up in triplicate in 10 ml GC-MS vials containing 2 ml fresh defined media (with 1 % w/v arabinose and appropriate antibiotics) and 40 μl of 0.1 g WCW/ml of either BDISC_0921, BDISC_0990, BDISC_0913 or BDISC_0664.

For the isoprene bioassay, an isoprene calibration series was set up in 10ml GC-MS vials containing 1990 μl defined media with 10 μl of 20 ppm to 1000 ppm of isoprene standards dissolved in 0.5 % v/v methanol at 4°C. To test isoprene assay robustness and precision, spike-recovery vials were also set up containing 10 μl of 1 ppm isoprene, for a random selection of the experimental conditions tested (one randomly selected strain for each NH_4Cl concentration set up in duplicate). All vials (isoprene/mevalonolactone experimental, isoprene standard and spike recovery vials) were incubated at 30 °C, 160 rpm, for 24 hours.

Example 5: Measurement of headspace isoprene concentrations

Headspace isoprene measurements were performed by GC-MS on an Agilent Technologies 7890B gas chromatograph connected

to an Agilent quadrupole 5977A MSD instrument with an electronically controlled split/split-less injection port. The instrument was equipped with a dual head MPS autosampler (Gerstel) for head Space analysis. GC separation was performed
 5 on a db-624 capillary column (60 m x 0.25mm x1.4µm J&W Scientific). The GC-MS parameters were as described in table 2. The M-1 ion was used for isoprene quantification.

Table 2. GCMS parameters for the measurement of head space isoprene concentrations

10

GCMS CONDITIONS		
PARAMETER	VALUE	
Carrier Gas	Helium at constant flow (2.0ml/min)	
Injector	Split ratio	Split 10:L
	Temperature	150 °C
Detector	Source Temperature	230 °C
	Quad Temperature	150 °C
	Interface	260 °C
	Gain	1
	Scan Range	m/z 28-200
	Threshold	150
	Scan Speed 2 ² (A/D samples) Sampling Rate 2 ⁿ =2 ²	4
	Mode	SCAN and SIM
Solvent delay *	5.50min	
Oven Temperature	Initial T: 40 °C x 6.9 min	
Oven Ramp	120 °C/min to 260 °C for 6 min	
Injection volume	500µl from the HS in the GC 2ml vial	
Incubation time and T	13 min at 95°C	
Agitator	ON 500 rpm	
Injection volume	500 µl of the Head Space	
Gas saver	On after 2 min	

Concentration range ($\mu\text{g/ml}$)	0.1-5.0
GC Column	DB-624 122-1334 Agilent (60m x 250 μm x 1.4 μm)

Example 6: Measurement of culture mevalonolactone concentrations

Culture broths were clarified by centrifugation 10,000 g for 10 minutes. The resulting supernatant (0.4 ml) was

5 acidified with 0.2 mL 0.5 M HCl, vortex for 10s and agitated at 1400 rpm for 15 minutes to convert all the mevalonic acid into mevalonolactone. The mevalonolactone was extracted from the aqueous phase by the addition of 0.5 ml of MTBE ((Methyl tertiary butyl ether) and the samples were wortex for 10s

10 agitated at 1400 rpm for a further 30 minutes. The MTBE used for the extraction contained an internal standard, carophyllene, at a concentration 10 ppm, for data normalisation (Pitera et al., 2006). A Thermomixer from Eppendorf was used for the 2 agitation spets. A

15 mevalonolactone calibration series was set up by a diluting mevalonolactone standard in 400 μl of defined media to final concentrations ranging from 1.5625 ppm to 100 ppm. To test assay robustness, spike recovery samples were set up with selected samples. All samples, including the standards were

20 treated in the same way. Following extraction, but leaving both phases in the same vial, 2 μl of the top layer was injected onto the GCMS. The GCMS parameters used to measure mevalonolactone are presented in Table 3. The presence of mevalonolactone in samples was confirmed by comparison of

25 retention time and ion ratios (Table 4) to those of the mevalonolactone standards, with the ion - 43 m/z being selected for quantification. All data from standards and

samples were normalised to the internal standard caryophyllene (selected ion - 93 m/z).

Table 3. GCMS parameters for the measurement of culture mevalonolactone concentrations

GCMS CONDITIONS		
PARAMETER	VALUE	
Carrier Gas	Helium at constant flow (1.0ml/min)	
Injector	Split ratio	Split less
	Temperature	230 °C
Detector	Source Temperature	230 °C
	Quad Temperature	150 °C
	Interface	270 °C
	Gain	2
	Scan Range	m/z 30-300
	Threshold	150
	Scan Speed 2 ² (A/D samples) Sampling Rate 2 ⁿ =2 ²	4
	Mode	SCAN and SIM
Solvent delay *	7.0min	
Oven Temperature	Initial T: 90 °C x 2min	
Oven Ramp	60 °C/min to 260 °C for 12 min	
Injection volume	2µl from the op organic layer in the 2ml GC vial	
Gas saver	On after 2 min	
Concentration range (µg/ml)	1.5625-100	
GC Column	DB-624 (60m x 250m x 1.4µm)	

5

Ions used for analysis and quantification of mevalonolactone and the internal standard (caryophyllene) in selected ion monitoring (SIM) acquisition mode are listed in Table 4.

Table 4. Ions used for mevalonolactone and the internal standard (caryophyllene) for quantitation in selected ion monitoring (SIM) acquisition mode

Compound	Ions monitored in SIM Mode (m/z)
Mevalonolactone	43, 58, 71
Internal standard - Caryophyllene	79, 91, 133

5

Example 7: Preparation of l-MVA variants

Chemically competent cells were transformed with variants of the l-MVA pathway which were designed to be arranged as single operons under the control of the wild type *P_{BAD}* promoter. The DNA assembly protocol used for this purpose was an optimized single-step overlap-extension PCR strategy. All operons contained the following genes in this specific order: i) mevalonate kinase (MVK), ii) mevalonate phosphokinase (MPK) and iii) mevalonate decarboxylase (MDD). Specifically, PCR products corresponding to each of the individual genes (MK, MPK and MDD, polypeptides of SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 and nucleic acid sequences of SEQ ID NO: 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44) and flanked by appropriately designed overlaps introduced by PCR were gel-extracted and suspended in dH₂O at a concentration of 1 ng/μl. One microliter of each of these DNA solutions was added (i.e. 3 μl in total) as DNA template to a PCR reaction, and a standard PCR procedure was carried out.

For the assembly of the combinatorial library the same strategy was used, but all the 17 different genes were pooled together simultaneously in a single 1.5 ml Eppendorf tube, for a total DNA concentration of approx. 30 ng/μl (about 1.8 ng/μl

each gene). One microliter of this solution was used as template DNA in a standard PCR reaction, and gel extraction was performed to isolate amplicons of fragment size of between 2.5 and 3.5 kb approximately. Gel-extracted DNA was digested
5 with AscI and SpeI and cloned.

The activity of the assembled l-MVA pathway variants was assessed by a functional assay providing indirect information about *E. coli* cellular synthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), two essential
10 metabolites used as terpenoids building blocks and synthesized as the last products of the l-MVA pathway (or the MEP pathway).

The antibiotic fosmidomycin (inhibitor of DOXP reductase (*dxr*), the second enzyme of the MEP pathway) was selected to
15 inhibit the native *E. coli* MEP pathway that supplies the essential metabolites IPP and DMAPP. NEB5 α competent cells were transformed with pBDISC0768-derivatives expressing the different l-MVA pathway variants, and then plated on LBA supplemented with tetracycline (10 μ g/ml), fosmidomycin (5
20 μ g/ml), and 1 mM (R)-mevalonic acid or (R)-Mevalonolactone. (R)-Mevalonolactone produced the same results as the corresponding acid form (R)-mevalonic acid (also in the isoprene detection assay described below), and it was selected as the substrate throughout both assays. As cells expressing a
25 functional l-MVA pathway can grow in the presence of inhibitory concentrations of fosmidomycin only when an appropriate concentration of (R)-mevalonate is also provided, this type of medium was specifically selective for functional l-MVA pathway variants from a library.

Isoprene production in the l-MVA pathway variants was also assessed. Specifically, pre-cultures of all *C. necator* strains tested were prepared in 20 ml TSB-D supplemented with appropriate antibiotics (tetracycline 10 ng/ μ l, kanamycin 200 ng/ μ l). After 48 hours of growth at 30°C, these pre-cultures were used to inoculate (2% v/v) 10 ml of the same medium and grown ON at 30°C at 230 rpm in a shaking incubator (induction with 1% (w/v) arabinose after 6-8 hours of growth).

The next day cultures were centrifuged and the pellet was re-suspended in new TSB-D plus antibiotic/inducer at a concentration of 0.1 g wcw/ml (wet cell weight/ml).

Gas chromatography vials (screw cap headspace gas chromatography (GC) vials (Anatune 093640-040-00 and 093640-038-00)) were prepared that contained with 1.96 ml of TSB-D supplemented with appropriate antibiotics as above, arabinose 1%, and 15 mM (R)-mevalonolactone, and inoculated with 40 μ l of the respective ON cell culture. All samples and 5 additional positive controls (containing standard concentration of isoprene) were prepared in triplicate and incubate 24h at 30°C (160rpm), before being analysed by gas chromatography-mass spectrometry using an Agilent GCMS 7890B -5977A with a Gerstel MPS Autosampler, set with the parameters reported in the following two tables Table A and Table B.

Table A

GCMS CONDITIONS	
PARAMETER	VALUE
Carrier Gas	Helium at constant flow (2.0ml/min)

Injector	Split ratio	Split 200:1
	Temperature	150 °C
Detector	Source Temperature	230 °C
	Quad Temperature	150 °C
	Interface	260 °C
	Gain	1
	Scan Range	m/z 28-200
	Threshold	150
	Scan Speed 2 ² (A/D samples) Sampling Rate 2 ⁿ =2 ²	4
	Mode	SCAN and SIM
Solvent delay *	5.50min	
Oven Temperature	Initial T: 40 °C x7.5min	
Oven Ramp	120 °C/min to 260 °C for 5 min	
Injection volume	300µl from the HS in the GC 2ml vial	
Incubation time and T	15 min at 95°C	
Agitator	ON 500 rpm	
Injection volume	300µl of the Head Space	
Gas saver	On after 2 min	
Concentration range (µg/ml)	25-250PPM	

GC Column	DB-624 122-1334 Agilent) 60m x 250 µm x 1.4 µm
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Table B

Compound	Ions monitored in SIM Mode (m/z)
Isoprene	39, 53, 67

Ions used for the Isoprene quantitation in selected ion monitoring (SIM) acquisition mode

5 **Example 8: Preparation of u-MVA variants**

The selection of genes used to prepare u-MVA is provided in Table 5.

Table 5: Genes used to perform the uMVA screen, with SEQ ID NO, organism of origin, gene function and GenBank/Uniprot identifier provided.

SEQ ID NO (nucleo-tide)	SEQ ID NO (amino acid)	Organism	Gene	GenBank / UniProt
55	56	<i>C. necator</i>	Acetoacetyl-CoA C-acetyltransferase	CAJ92573
67	68	<i>Saccharomyces cerevisiae</i>	Hydroxymethylglutaryl-CoA synthase	NP_013580
69	70	<i>Listeria monocytogenes</i>	hydroxymethylglutaryl-CoA synthase	WP_003732325
71	72	<i>Lactococcus lactis</i>	Hydroxymethylglutaryl-CoA synthase	WP_010906037
57	58	<i>Saccharomyces cerevisiae</i>	Hydroxymethylglutaryl-CoA reductase (Residues 554-1054)	AJS96703
59	60	<i>Listeria monocytogenes</i>	Hydroxymethylglutaryl-CoA reductase	WP_003721392
61	62	<i>Streptococcus pneumoniae</i>	Hydroxymethylglutaryl-CoA reductase	WP_000704390
63	64	<i>Lactococcus lactis</i>	Hydroxymethylglutaryl-CoA reductase	WP_010906035
65	66	<i>Staphylococcus aureus</i>	Hydroxymethylglutaryl-CoA reductase	WP_072512079.1
73	2	<i>Enterococcus faecalis</i>	mvaS mutant, Hydroxymethylglutaryl-CoA synthase, with A110G mutation	2HDB_A
23	1	<i>Enterococcus faecalis</i>	mvaE (bifunctional enzyme with acetoacetyl-CoA C-acetyltransferase and hydroxymethylglutaryl-CoA	WP_002382276

uMVA pathway library cloning strategy

The uMVA pathway library was constructed by assembling a set of synthetic operons, under the control of the arabinose-inducible P_{BAD} promoter, with the following order of DNA parts:

5 P_{BAD} promoter-ISPS-PhaA-HMGR-HMGS-rrrnBT2 terminator. (ISPS = isoprene synthase; PhaA = acetoacetyl-CoA C-acetyltransferase; HMGR= hydroxymethylglutaryl-CoA reductase and HMGS = hydroxymethylglutaryl-CoA synthase).

10 Individual genes were amplified by PCR and cloned using standard molecular biology techniques. The different operons assembled are listed in Table 6. The constructs were transformed into chemically competent *E. coli* cells and correct clones were verified by colony PCR. Plasmids were recovered, and the presence of the insert was confirmed by
15 sequencing. The correct constructs were transformed in *C. necator* and new strains were assessed by their ability to generate isoprene.

Table 6: Description of uMVA constructs generated.

uMVA variant	Description
uMVA_01	pBBR1-1A-pBAD- <i>Salix</i> ISPS- <i>C.necator</i> _phaA- <i>S.cerevisiae</i> HMGR - <i>S.cerevisiae</i> HMGS-rrnBt2
uMVA_02	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S.cerevisiae</i> HMGR - <i>L. monocytogenes</i> HMGS-rrnBt2
uMVA_03	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S.cerevisiae</i> HMGR - <i>L.lactis</i> HMGS-rrnBt2
uMVA_04	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S.cerevisiae</i> HMGR - <i>E. faecalis</i> mvaS mutant-rrnBt2
uMVA_05	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L. monocytogenes</i> HMGR - <i>S.cerevisiae</i> HMGS -rrnBt2
uMVA_06	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L. monocytogenes</i> HMGR - <i>L. monocytogenes</i> HMGS -rrnBt2
uMVA_07	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L. monocytogenes</i> HMGR - <i>L.lactis</i> HMGS -rrnBt2
uMVA_08	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L.</i>

uMVA variant	Description
	<i>monocytogenes</i> HMGR - <i>E. faecalis</i> mvaS mutant rrnBt2
uMVA_13	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. pneumoniae</i> HMGR- <i>S.cerevisiae</i> HMGS -rrnBt2
uMVA_14	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. pneumoniae</i> HMGR - <i>L. monocytogenes</i> HMGS -rrnBt2
uMVA_15	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. pneumoniae</i> HMGR - <i>L.lactis</i> HMGS -rrnBt2
uMVA_16	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. pneumoniae</i> HMGR - <i>E. faecalis</i> mvaS mutant-rrnBt2
uMVA_17	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L.lactis</i> HMGR - <i>S.cerevisiae</i> HMGS -rrnBt2
uMVA_18	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L.lactis</i> HMGR - <i>L. monocytogenes</i> HMGS -rrnBt2
uMVA_19	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L.lactis</i> HMGR - <i>L.lactis</i> HMGS -rrnBt2
uMVA_20	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>L.lactis</i> HMGR - <i>E. faecalis</i> mvaS mutant-rrnBt2
uMVA_21	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. aureus</i> HMGR- <i>S.cerevisiae</i> HMGS -rrnBt2
uMVA_22	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. aureus</i> HMGR - <i>L. monocytogenes</i> HMGS -rrnBt2
uMVA_23	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. aureus</i> HMGR - <i>L.lactis</i> HMGS -rrnBt2
uMVA_24	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>C.necator</i> _phaA- <i>S. aureus</i> HMGR - <i>E. faecalis</i> mvaS mutant-rrnBt2
uMVA_25	pBBR1-1A-pBAD- <i>Salix</i> ISPS - <i>E. faecalis</i> mvaE- <i>E. faecalis</i> mvaS -rrBt2

Isoprene Assay

To assess isoprene production in a *C. necator* strain with the whole MVA pathway and an isoprene synthase, uMVA-pathway variants were expressed in a *C. necator* H16 derivative strain. This strain possesses the Δ phaC1AB1 and Δ H16_A0006-9 genotypes conferring a PHA- phenotype and improved transformation efficiency, respectively. This strain also has an *ispS* (encoding the isoprene synthase of *P. alba*) and the *E. faecalis* uMVA construct integrated into chromosome 1 at the

phaC1AB1 locus. It also has the *S. pneumonia* lMVA-idi construct integrated into chromosome 1 at the *phaB2C2* locus. Expression of the plasmid-based uMVA-pathway variants in this genetic background supplemented the activity conferred by the integrated *E. faecalis* uMVA construct. Therefore, it was possible to assess the impact (if any) of the different uMVA pathways on isoprene production.

C. necator library strains were evaluated for *de novo* isoprene synthesis in bioassays based on gas chromatography-mass spectrometry (GC-MS). Seeding cultures were prepared for each strain by inoculating a single colony into 20 ml of 27.5 g/L Tryptone Soya Broth without Dextrose containing the appropriate antibiotic when necessary. Unless otherwise stated, the seeding cultures were incubated at 30°C, 230rpm for 48 hours, then diluted by 1 in 50 into fresh TSB-D media (10 ml) in 50 ml sterile Falcon tubes and incubated for approximately 6-7 hours at 30°C, 230rpm. MVA pathway and isoprene synthase expression was induced by adding arabinose to a final concentration of 0.4- 1 % w/v and the cultures were incubated for a further 16 hours (overnight) at 30°C, 230rpm. The cultures were pelleted by centrifugation at 6000 *g* for 20 minutes and the density of each culture was normalized to either a standard optical density (OD₆₀₀) or wet cell weight (WCW) concentration (gWCW/ml) by re-suspending the *C. necator* cells with the appropriate volume of either minimal media or TSB media. For each experimental variable (strain and media conditions), separate isoprene bioassays were set up in triplicate in 10 ml GC-MS vials containing 2 ml fresh minimal or TSB media (with 1 % w/v arabinose and appropriate antibiotics) and 40 µl- 60 µl of each cell suspension.

An isoprene calibration series was set up in 10 ml GC-MS vials containing 1990 µl minimal/TSB media with 10 µl of 20 ppm to 1000 ppm of isoprene standards dissolved in 0.5 % v/v methanol at 4°C. To test isoprene assay robustness and precision, spike-recovery vials were also set up containing 10 µl of 1 ppm isoprene, for a random selection of the experimental conditions tested. All vials (isoprene experimental, isoprene standard and spike recovery vials) were incubated at 30 °C, 160 rpm, for 24 hours.

10 **Measurement of headspace isoprene concentrations**

Headspace isoprene measurements were performed by GC-MS on an Agilent Technologies 7890B gas chromatograph connected to an Agilent quadrupole 5977A MSD instrument with an electronically controlled split/split-less injection port. The instrument was equipped with a dual head MPS autosampler (Gerstel) for head Space analysis. GC separation was performed on a db-624 capillary column (60 m x 0.25mm x1.4µm J&W Scientific). The GC-MS parameters were as described in Table 7. The M-1 ion was used for isoprene quantification.

20 **Table 7. Typical GCMS parameters for the measurement of head space isoprene concentrations.**

GCMS CONDITIONS	
PARAMETER	VALUE
Carrier Gas	Helium at constant flow (2.0ml/min)
Injector	Split ratio Split 10:L Temperature 150 °C

Detector	Source Temperature Quad Temperature Interface Gain Scan Range Threshold Scan Speed 2 ² (A/D samples) Sampling Rate 2 ⁿ =2 ² Mode	230 °C 150 °C 260 °C 1 m/z 28-200 150 4 Rate SCAN and SIM
Solvent delay *	5.50min	
Oven Temperature	Initial T: 40 °C x 6.9 min	
Oven Ramp	120 °C/min to 260 °C for 6 min	
Injection volume	500µl from the HS in the GC 2ml vial	
Incubation time and T	13 min at 95°C	
Agitator	ON 500 rpm	
Injection volume	500 µl of the Head Space	
Gas saver	On after 2 min	
Concentration range (µg/ml)	0.1-5.0	
GC Column	DB-624 122-1334 Agilent (60m x 250 µm x 1.4 µm)	

A total of 11 strains yielded more isoprene than the benchmark strain (harbouring a plasmid expressing the *Salix* ISPS) with uMVA_22 and uMVA_21 variants producing highest isoprene
5 titers. Nine variants are shown in Table 8 which show isoprene accumulation of more than 200 ppm.

Table 8: Isoprene accumulation in BDISC0921 in parts per million (ppm) and peak area (response*). Values represent average of 3 technical replicas (AV) and respective standard deviation (SD). Strains are ranked according to response. BM, Benchmark strain is BDISC1079; ISPS is a negative control. Calibration curve: 10-100ppm (linearity confirmed until 200ppm).

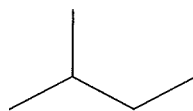
Sample #	Plasmids	ISPS	Thiolase (phaA)	HMG-Reductase	HMG-Synthase	Isoprene [ppm]		Response	
						Av	SD	Av	SD
uMVA_22	pBBR1-1A- pBAD-ISPS- phaA-HMGR- HMGS	<i>Salix</i>	<i>C. necator</i>	<i>S. aureus</i>	<i>L. monocytogenes</i>	>20	N/A	10688 202	3640 67
uMVA_21	pBBR1-1A- pBAD-ISPS- phaA-HMGR- HMGS	<i>Salix</i>	<i>C. necator</i>	<i>S. aureus</i>	<i>S. cerevisiae</i>	>20	N/A	78718 10	1245 912
uMVA_02	pBBR1-1A- pBAD-ISPS- phaA-HMGR- HMGS	<i>Salix</i>	<i>C. necator</i>	<i>S. cerevisiae</i>	<i>L. monocytogenes</i>	>20	N/A	77173 83	2491 89
uMVA_05	pBBR1-1A- pBAD-ISPS- phaA-HMGR- HMGS	<i>Salix</i>	<i>C. necator</i>	<i>L. monocytogenes</i>	<i>S. cerevisiae</i>	>20	N/A	74197 99	5257 28
uMVA_06	pBBR1-1A- pBAD-ISPS- phaA-HMGR- HMGS	<i>Salix</i>	<i>C. necator</i>	<i>L. monocytogenes</i>	<i>L. monocytogenes</i>	>20	N/A	74153 79	1659 318
uMVA_03	pBBR1-1A-	<i>Salix</i>	<i>C.</i>	<i>S.</i>	<i>L. lactis</i>	>20	N/A	71187	7137

	pBAD- ISPS- phaA-HMGR- HMGS		necator	cerevisia e		0		50	24
uMVA_01	pBBR1-1A- pBAD- ISPS- phaA-HMGR- HMGS	Salix	C. necator	S. cerevisia e	S. cerevisiae	>20 0	N/A	64158 93	8718 19
uMVA_13	pBBR1-1A- pBAD- ISPS- phaA-HMGR- HMGS	Salix	C. necator	S. pneumonia e	S. cerevisiae	>20 0	N/A	53232 52	7055 10
uMVA_14	pBBR1-1A- pBAD- ISPS- phaA-HMGR- HMGS	Salix	C. necator	S. pneumonia e	L. monocyto nes	>20 0	N/A	35228 66	4265 28
BM	pBBR1-1A- pBAD- ISPS- phaA-HMGR- HMGS	Salix	-	-	S. cerevisiae	105 .34 2	55. 417	17124 51	9008 67
ISPS	pBBR1-1A- pBAD-407	Salix	-	-	-	1.7 35	0.6 74	28212	1095 6

What is Claimed is:

1. A genetically engineered host capable of producing hydrocarbons via a mevalonate (MVA) pathway.

5 2. The genetically engineered host of claim 1, wherein said hydrocarbon comprises one or more isoprene units as depicted in Formula I



(I)

or a salt or derivative thereof.

10

3. The genetically engineered host of claim 1 or claim 2 comprising at least one genome-integrated synthetic operon encoding an enzyme of the MVA pathway.

15 4. The genetically engineered host of claim 1 or claim 2 comprising a genome-integrated synthetic operon encoding a plurality of enzymes of the MVA pathway.

5. The genetically engineered host of any of the
20 preceding claims encoding one or more enzymes selected from acetoacetyl-CoA C-acetyltransferase (AACT), HMG-CoA reductase (HMGR), hydroxymethylglutaryl-CoA synthase (HMGS), mevalonate kinase (MVK), phosphomevalonate kinase (MPK0, mevalonate diphosphate decarboxylase (MDD), isopentenyl diphosphate
25 isomerase (IDI) and isoprene synthase (ISPS).

6. The genetically engineered host of any of the preceding claims comprising a genome-integrated synthetic

operon encoding one or more of enzymes of the upper MVA pathway.

7. The genetically engineered host of any of the
5 preceding claims comprising a genome-integrated synthetic operon encoding one or more of the enzymes of the *Enterococcus faecalis* upper MVA pathway.

8. The genetically engineered host of claim 6 or 7
10 comprising an exogenous nucleic acid sequence encoding a polypeptide having AACT, HMGR or HGMS enzyme activity.

9. The genetically engineered host of claim 8 wherein the polypeptide having AACT, HMGR or HGMS enzyme activity has at least 70% sequence identity to an amino acid sequence set
15 forth in SEQ ID NO: 1, 56, 58, 60, 62, 64 66, 2, 68, 70, 72 or 74 or a functional fragment thereof.

10. The genetically engineered host of claim 8 wherein the polypeptide having AACT, HMGR or HGMS enzyme activity comprises an amino acid sequence set forth in SEQ ID NO: 1,
20 56, 58, 60, 62, 64 66, 2, 68, 70, 72 or 74 or a functional fragment thereof.

11. The genetically engineered host of claim 8 wherein the exogenous nucleic acid sequence has at least 70% sequence identity to the nucleic acid sequence set forth in SEQ ID NO:
25 23, 55, 57, 59, 61, 63, 65, 24, 67, 69, 71 or 73 or a functional fragment thereof.

12. The genetically engineered host of claim 8 wherein the exogenous nucleic acid sequence comprises SEQ ID NO: 23, 55, 57, 59, 61, 63, 65, 24, 67, 69, 71 or 73 or a functional fragment thereof.

5 13. The genetically engineered host of any of the preceding claims comprising a genome-integrated synthetic operon encoding one or more enzymes of the lower MVA pathway.

10 14. The genetically engineered host of claim 13 comprising a genome-integrated synthetic operon encoding one or more enzymes of the *Streptococcus pneumoniae* lower MVA pathway.

15 15. The genetically engineered host of claim 13 or 14 comprising an exogenous nucleic acid sequence encoding a polypeptide having mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase or isopentenyl diphosphate isomerase enzyme activity.

20 16. The genetically engineered host of claim 15 wherein the polypeptide having mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase or isopentenyl diphosphate isomerase enzyme activity has at least 70% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 3, 4, 5 or 6 or a functional fragment thereof.

25 17. The genetically engineered host of claim 15 wherein the polypeptide having mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase or isopentenyl diphosphate isomerase enzyme activity comprises an amino acid sequence set forth in SEQ ID NO: 3, 4, 5 or 6 or a functional fragment thereof.

18. The genetically engineered host of claim 15 wherein the exogenous nucleic acid sequence has at least 70% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 25, 26, 27 or 28 or a functional fragment thereof.

5 19. The genetically engineered host of claim 15 wherein the exogenous nucleic acid sequence comprises SEQ ID NO: 25, 26, 27 or 28 or a functional fragment thereof.

20. The genetically engineered host of any of claims 1-5 comprising a genome-integrated synthetic operon encoding
10 polypeptides having mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) enzyme activities.

21. The genetically engineered host of claim 20 comprising exogenous nucleic acid sequences encoding
15 polypeptide having MK, MPK and MDD enzyme activities.

22. The genetically engineered host of claim 21 wherein the polypeptides having MK, MPK and MDD enzyme activity have at least 70% sequence identity to an amino acid sequence set forth in SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,
20 20, 21 or 22 or a functional fragment thereof.

23. The genetically engineered host of claim 21 wherein the polypeptides having MK, MPK and MDD enzyme activity comprise amino acid sequences set forth in SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 or a
25 functional fragment thereof.

24. The genetically engineered host of claim 21 wherein the exogenous nucleic acid sequences have at least 70% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43
5 or 44 or a functional fragment thereof.

25. The genetically engineered host of claim 21 wherein the exogenous nucleic acid sequences comprise SEQ ID NO: 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44 or a functional fragment thereof.

10 26. The genetically engineered host of any of claims 1-5 comprising a genome-integrated synthetic operon comprising SEQ ID NO: 48, 49 or 50.

27. The genetically engineered host of any of claims 1 through 26 further comprising a genome-integrated
15 transcription unit coding for an isoprene synthase enzyme.

28. The genetically engineered host of claim 27 wherein the isoprene synthase enzyme comprises a polypeptide having at least 70% sequence identity to an amino acid sequence set forth in SEQ ID NOs: 7 or 8 or a functional fragment thereof.

20 29. The genetically engineered host of claim 27 wherein the isoprene synthase enzyme comprises SEQ ID NOs: 7 or 8 or a functional fragment thereof.

30. The genetically engineered host of claim 27 wherein the isoprene synthase enzyme is encoded by a nucleic acid
25 sequence having at least 70% sequence identity to SEQ ID NOs: 29 or 30 or a functional fragment thereof.

31. The genetically engineered host of claim 27 wherein the isoprene synthase enzyme is encoded by a nucleic acid sequence comprising SEQ ID NOs: 29 or 30 or a functional fragment thereof.

5 32. The genetically engineered host of any of the preceding claims further comprising one or more plasmids encoding one or more enzymes of the lower and/or upper MVA pathways.

10 33. The genetically engineered host of any of the preceding claims having one or more properties of *Cupriavidus necator*.

15 34. The genetically engineered host of any of the preceding claims selected from non-pathogenic members of the genera *Ralstonia*, *Wauteria*, *Cupriavidus*, *Alcaligenes*, *Burkholderia* or *Pandoraea*.

35. The recombinant host according to any of the preceding claims which is *Cupriavidus necator*.

20 36. A method for producing a genetically engineered host which produces hydrocarbons, said method comprising integrating at least one synthetic operon encoding one or more enzymes of the MVA pathway into the host genome.

25 37. The method of claim 36 wherein the synthetic operon encodes a plurality of enzymes of the MVA pathway.

38. The method of claim 36 wherein the synthetic operon encodes one or more enzymes selected from acetoacetyl-CoA C-acetyltransferase (AACT), HMG-CoA reductase (HMGR),

hydroxymethylglutaryl-CoA synthase (HMGS), mevalonate kinase (MVK), phosphomevalonate kinase (MPK), mevalonate diphosphate decarboxylase (MDD), isopentenyl diphosphate isomerase (IDI) and isoprene synthase (ISPS).

5

39. The method of claim 36 comprising integrating a synthetic operon encoding the *Enterococcus faecalis* upper MVA pathway into the host genome.

10

40. The method of claim 36 or claim 39 comprising integrating a synthetic operon encoding *Streptococcus pneumoniae* lower MVA pathway into the host genome.

15

41. The method of claim 36 comprising integrating a synthetic operon encoding polypeptides having mevalonate kinase (MK), mevalonate phosphokinase (MPK), and mevalonate decarboxylase (MDD) enzyme activities.

20

42. The method of any of claims 36 through 41 further comprising integrating a transcription unit coding for an isoprene synthase into the host genome.

25

43. The method of any of claims 36 through 42 further comprising incorporating one or plasmids encoding one or more enzymes of the upper and/or lower MVA pathways into the host.

44. A bioderived isoprenoid produced in a genetically engineered host according to any of claims 1 through 35.

45. A bio-derived, bio-based, or fermentation-derived product produced from any of the hosts of any of claims 1 to 35, wherein said product comprises:

(i) a composition comprising at least one bio-derived, bio-based, or fermentation-derived compound or any combination thereof;

(ii) a bio-derived, bio-based, or fermentation-derived polymer comprising the bio-derived, bio-based, or fermentation-derived composition or compound of (i), or any combination thereof;

(iii) a bio-derived, bio-based, or fermentation-derived cis-polyisoprene rubber, trans-polyisoprene rubber, or liquid polyisoprene rubber, comprising the bio-derived, bio-based, or fermentation-derived compound or bio-derived, bio-based, or fermentation-derived composition of (i), or any combination thereof or the bio-derived, bio-based, or fermentation-derived polymer of (ii), or any combination thereof;

(iv) a molded substance obtained by molding the bio-derived, bio-based, or fermentation-derived polymer of (ii), or the bio-derived, bio-based, or fermentation-derived rubber of (iii), or any combination thereof;

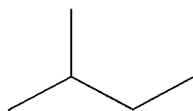
(v) a bio-derived, bio-based, or fermentation-derived formulation comprising the bio-derived, bio-based, or fermentation-derived composition of (i), the bio-derived, bio-based, or fermentation-derived compound of (i), the bio-derived, bio-based, or fermentation-derived polymer of (ii), the bio-derived, bio-based, or fermentation-derived rubber of (iii), or the bio-derived, bio-based, or fermentation-derived molded substance of (iv), or any combination thereof; or

(vi) a bio-derived, bio-based, or fermentation-derived semi-solid or a non-semi-solid stream, comprising the bio-

derived, bio-based, or fermentation-derived composition of (i), the bio-derived, bio-based, or fermentation-derived compound of (i), the bio-derived, bio-based, or fermentation-derived polymer of (ii), the bio-derived, bio-based, or fermentation-derived rubber of (iii), the bio-derived, bio-based, or fermentation-derived formulation of (iv), or the bio-derived, bio-based, or fermentation-derived molded substance of (v), or any combination thereof.

46. A method for synthesizing a hydrocarbon in a genetically engineered host of any of claims 1 through 35, said method comprising enzymatically converting mevalonate to isopentenyl-pyrophosphate in the host.

47. The method of claim 46, wherein said hydrocarbon comprises one or more isoprene units as depicted in Formula I



(I)

or a salt or derivative thereof.

48. The method of claim 46, wherein at least one of the enzymatic conversions comprises fermentation of a biological or non-biological feedstock.

49. The method of claim 46, wherein at least one of the enzymatic conversions comprises gas fermentation within the genetically engineered host.

50. The method of claim 49, wherein the gas fermentation comprises at least one of natural gas, syngas, CO₂/H₂, methanol, ethanol, non-volatile residue, caustic wash from cyclohexane oxidation processes, or waste stream from a
5 chemical or petrochemical industry.

51. The method of claim 49 wherein the gas fermentation comprises CO₂/H₂.

10

52. The method of any of claims 46 through 51, further comprising recovering produced hydrocarbon.

53. The method of claim 52 wherein the hydrocarbon
15 recovered is gaseous isoprene.

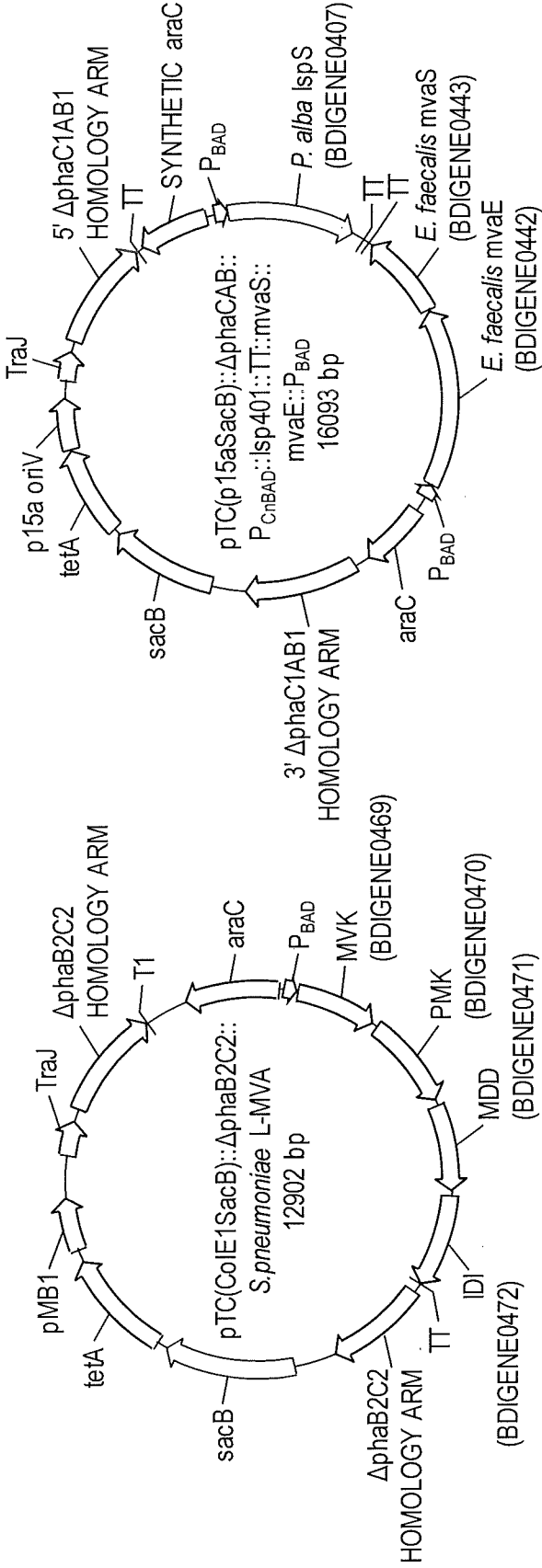


FIG. 1B

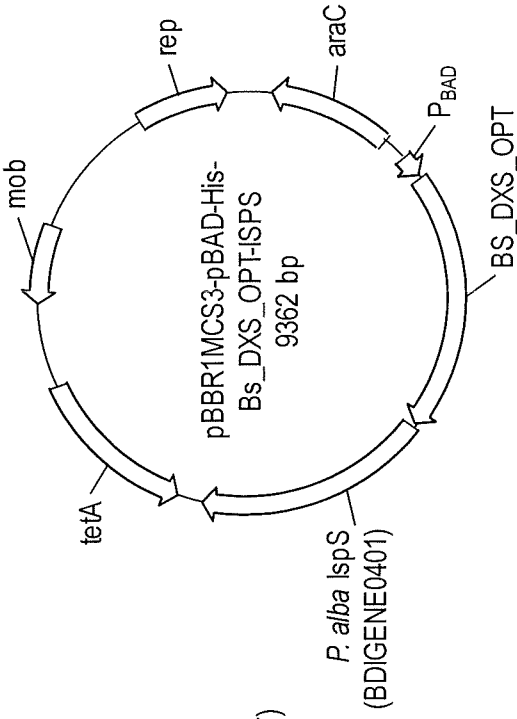


FIG. 1D

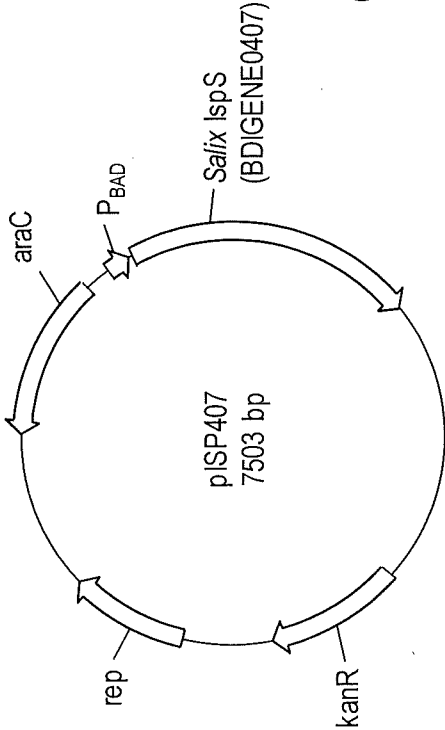


FIG. 1C

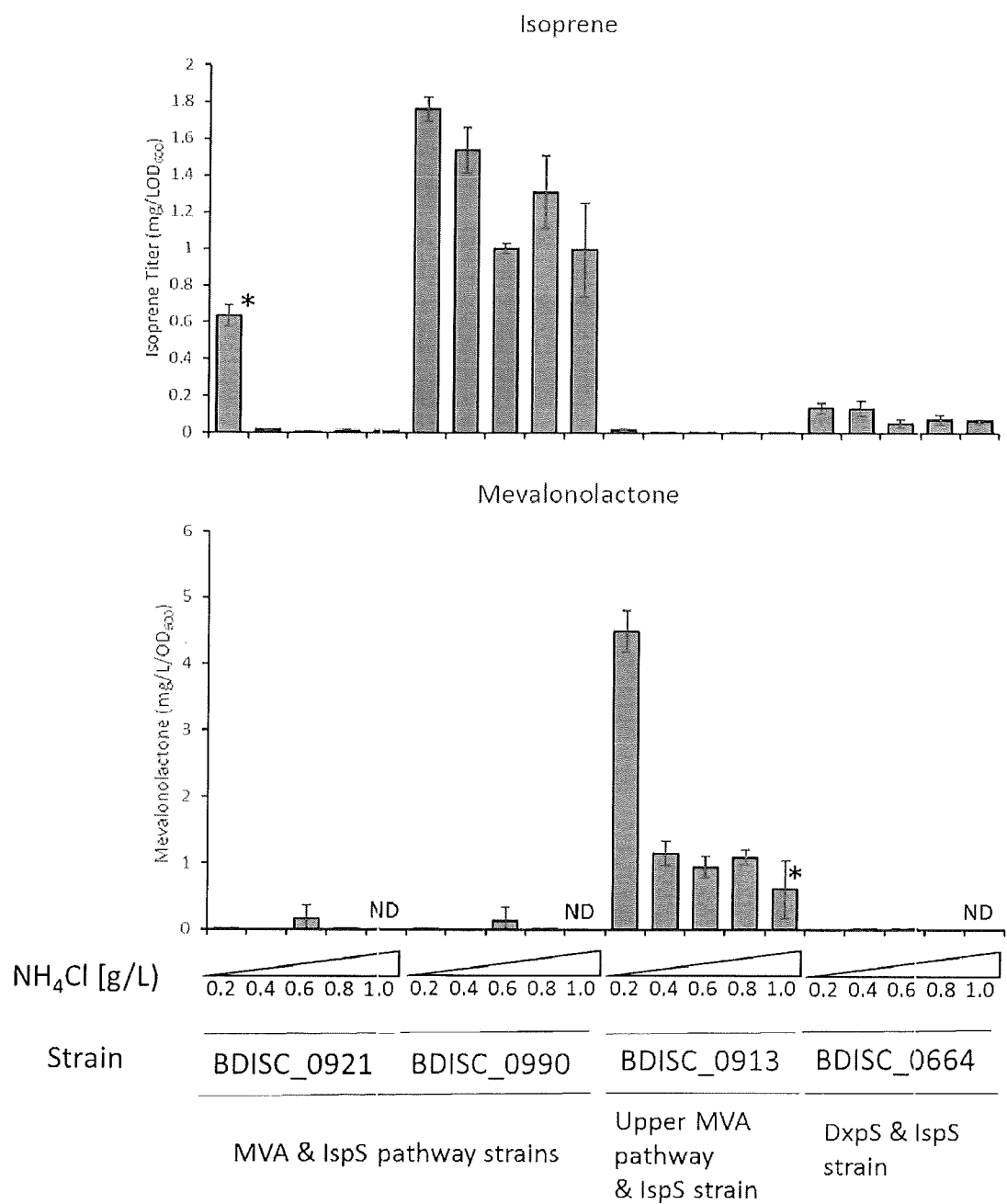
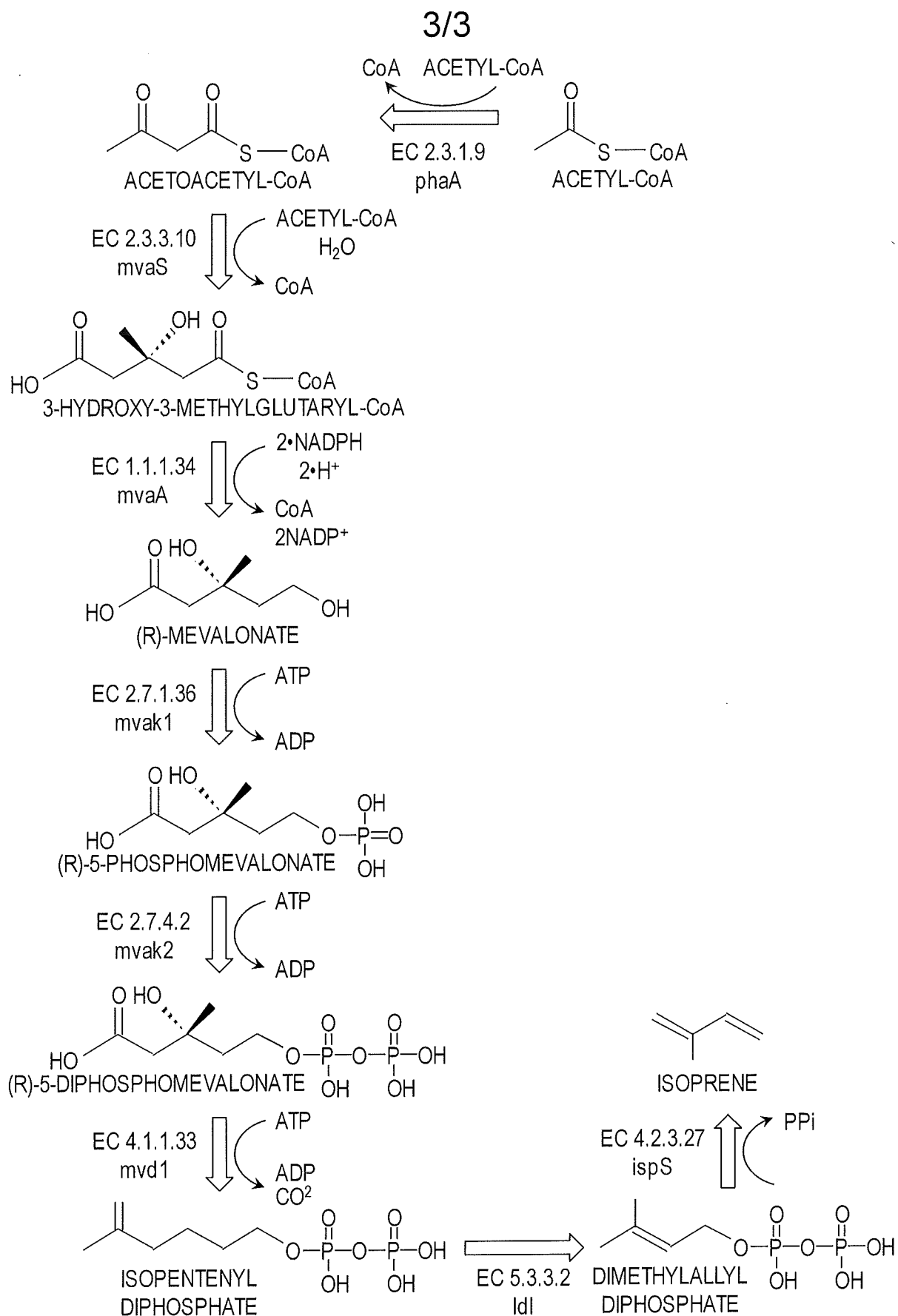


FIG. 2

**FIG. 3**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040213

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07C 11/18; C07F 9/09; C08F 136/08; C12N 1/15 (2018.01)

CPC - C07C 11/18; C07F 9/09; C12P 9/00; C12Y 207/04026; C12Y 401/01 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/119; 435/123; 435/127; 435/131; 435/136 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0145441 A1 (INVISTA NORTH AMERICA S.À.R.L.) 25 May 2017 (25.05.2017) entire document	1-4, 36-41
A	US 2014/0335576 A1 (DANISCO US INC. et al) 13 November 2014 (13.11.2014) entire document	1-4, 36-41
A	US 2015/0284742 A1 (SEKISUI CHEMICAL CO., LTD. et al) 08 October 2015 (08.10.2015) entire document	1-4, 36-41
A	US 2016/0002672 A1 (DANISCO US INC. et al) 07 January 2016 (07.01.2016) entire document	1-4, 36-41
A	WO 2006/014837 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al) 09 February 2006 (09.02.2006) entire document	1-4, 36-41

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 August 2018

Date of mailing of the international search report

21 SEP 2018

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040213

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-35, 42-53
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.